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DETECTION AND QUANTIFICATION OF HERBICIDE RESIDUES IN THE ENVIRONMENT USING IMMUNOCHEMICAL TECHNIQUES

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Introduction to the Use of Immunoassays for the Detection and Quantitation of Herbicides in the Environment

Abstract. Immunochemical techniques offer many advantages over chromatographic methods currently used for pesticide trace analysis of substrates such as soil, water, plants, urine, and blood. These advantages include speed of processing samples, high specificity for detecting the pesticide of interest, reduced amount of preparation and cleanup of the sample prior to analysis, and a dramatic increase in the number of samples that can be analyzed. Immunoassays are based on the principle that antibodies to pesticides can be prepared, in animals, that can recognize and attach with equisite specificity to certain chemical configurations displayed on the surface of a molecule. Small molecules such as herbicides are not usually immunogenic but can be made so by chemically bonding them to a large immunogenic protein such as bovine serum albumin prior to injection into an animal. Development of herbicide specific antibodies and their use in direct and indirect enzyme linked immunosorbent assays (EIA or ELISA) as well as radioimmunoassays (RIA) are discussed. The principles behind monoclonal antibody production are outlined and immunoassays using polyclonal and monoclonal antibodies are compared. Specific reference is made to the development and use of indirect ELISA and RIA procedures for trace analysis of 2,4-D and picloram.

INTRODUCTION

As we approach the 1990's, public awareness of environmental issues is increasing. People are concerned about the fate of industrial pollutants and the risks of exposure to chemicals used in agriculture and other industries. Organizations such as the Environmental Protection Agency, USDA, Agriculture Canada, and Health and Welfare Canada have been aware for some time of the risks and benefits of pesticides used in agriculture and forestry. As public concern about the pesticide issue continues to increase, the pressure to provide new information and guidelines on the fate of pesticides in the environment has become a monumental task for governments and industry. For example, industry requires more detailed information for pesticide registration in Canada and the

United States, and government and university organizations are required to provide critical evaluations on the fate of pesticides in the soil, potable water, and food components.

Detection of pesticides has been based mainly on conventional techniques such as gas-liquid, high-performance liquid, and thin-layer chromatographic methods. Although these techniques are sensitive and reproducible, they are cumbersome, time consuming, and extremely costly. Thus, there is a clear need for simple, rapid, and inexpensive methods for the quantitative detection and measurement of pesticides in a variety of water, soil and plant materials. An approach to achieve this objective may be the utilization of detection methods based upon assays utilizing well-understood immunological principles.

In disciplines such as clinical chemistry and endocrinology, immunochemistry is often the analytical method of choice because of its sensitivity, specificity, speed of analysis, ease of automation, cost effectiveness, and general applicability. The potential of immunochemical technology for pesticide residue analysis in various substrates such as soil, water, plants, urine, and blood has been examined by several authors (1,2,3,7,8,11,15,16). Certain immunochemical pesticide assays have already been commercialized. With regard to immunochemical techniques for herbicide trace analysis, a review of the published literature citations is presented in Table 1.

WHAT IS AN IMMUNOASSAY

Antibodies belong to a class of protein molecules known as immunoglobulins. Immunoassays are based upon the fundamental principle that antibodies prepared in animals can recognize and attach with exquisite specificity to certain chemical configurations displayed on the surface of simple or complex molecules. In some cases, the specificity of this binding can be so great that, for example, antibodies specific for para-nitrophenol will not bind with ortho-nitrophenol. This is amazing considering that these two compounds have the same chemical formula but the elements making up the two isomers have a different spatial arrangement.

Table 1. Review of published immunoassays for herbicides.

Herbicide	Antibody	Method	Reference
Atrazine	P[a]	ELISA[b]	9
Chlorsulfuron	P	ELISA	12
2,4-D	P	RIA[c]	18
	P	RIA	13
	P	ELISA	5
	P	RIA	6
	P	ELISA	6
Diclofop-methyl	P	ELISA	20
	P	Fluoroimmunoassay	20
Paraquat	P	RIA	14
	P	RIA	4
	M[d]	ELISA	17
	P	ELISA	21
	P	ELISA	22
Picloram	P	RIA	6
Terbutryn	P	ELISA	10
2,4,5-T	P	RIA	18

[a] P = polyclonal antibody

[b] ELISA = enzyme-linked immunosorbent assay

[c] RIA = radioimmunoassay

[d] M = monoclonal antibody

Thus, by combining specific antibodies and an appropriate indicator system, the aforementioned specific binding can be used to identify the presence of a specific chemical at quantitative levels approaching 1 ppb or less without interference from closely related compounds.

Traditional methods of producing specific antibodies involve the immunization of experimental animals such as mice, rats, or rabbits with chemical compounds having a molecular weight which exceeds approximately 1 X 10^4 Dalton. Subsequently, an immunologic response occurs that is typified by the synthesis of antibodies specific for the immunizing molecule. In the case of proteins, the myriad of chemical structures displayed on the outer surface of the molecule will elicit the synthesis of an antibody specific for each unique structure.

Although small molecules alone are not usually capable of inducing antibody synthesis, such molecules can be chemically bonded to large protein molecules. Following immunization, the resulting antibodies will not only be specific for the protein surface structures, but they will also recognize the synthetically attached small molecule called the hapten. Thus, it is theoretically possible to prepare antibodies specific for low molecular weight molecules such as pesticides and drugs.

PRODUCTION OF HERBICIDE SPECIFIC POLYCLONAL ANTIBODIES

To produce the antibodies needed for a herbicide-specific immunoassay, the pesticide or plant growth regulator must be chemically coupled to a high molecular weight protein such as bovine serum albumin (BSA) to produce a herbicide/BSA conjugate (8). Animals such as New Zealand white rabbits are then injected subcutaneously with a buffered solution containing the herbicide/BSA conjugate. Because BSA is a large protein that is regarded as being foreign by the rabbits' immune system, antibodies are produced to the BSA. Antibodies specific against the herbicide also are produced because the herbicide is attached to the BSA.

The rabbits receive booster injections of herbicide/BSA 7, 21, 42, and 49 days after the initial injection. Several millilitres of blood are taken from the marginal vein of the ear two months after the initial herbicide/BSA injection and thereafter at monthly intervals. Serum is prepared from the blood by

removing the red blood cells. Antibodies present in the serum can be used directly for an immunoassay or can be purified from the serum by simple biochemical techniques such as protein precipitation and/or isolation of immunoglobulin G fractions using protein A columns.

DEVELOPMENT OF THREE BASIC IMMUNOASSAYS FOR THE DETECTION OF HERBICIDES

Three basic types of immunoassays can be used to detect herbicides and include i) indirect ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA), ii) direct ELISA, and iii) RADIOIMMUNOASSAY (RIA).

Indirect ELISA (Fig. 1)

A polystyrene microtiter plate containing 96 wells is coated with a herbicide coupled to an irrelevant carrier protein such as rabbit serum albumin (RSA) instead of BSA to avoid reactivity with the non-specific antibodies to BSA that are present in the serum. The properties of the microtiter plate are such that each well will passively bind any large protein molecule because of the electrostatic attraction between the protein and the well of the plate.

As mentioned earlier, most small molecules will not, by themselves, bind to the microtiter plate and therefore the herbicide is chemically bonded to an inert protein such as RSA. The RSA will bind to the plate leaving the attached herbicide molecule exposed for further reaction. RSA is chosen because the antibodies to the herbicide were raised in rabbits, therefore there is little chance that the rabbit serum will contain any antibodies that will react with the RSA bound to the wells of the plate.

After allowing the plates to stand at room temperature for 30 minutes, the wells of the plate are washed three times with buffer solution to remove any unbound herbicide/RSA.

Dilute serum containing the herbicide antibody is now added to each of the microtiter plate wells and the plate is allowed to incubate for one hour to allow the herbicide antibodies to bind to the herbicide molecules bound to each well. The wells are then washed with buffer to remove any excess antibody.

The herbicide antibodies bound to the wells are revealed by adding a second antibody specific for any rabbit immunoglobulin. The second antibody is raised by immunizing goats against immunoglobulins found in rabbits and is therefore

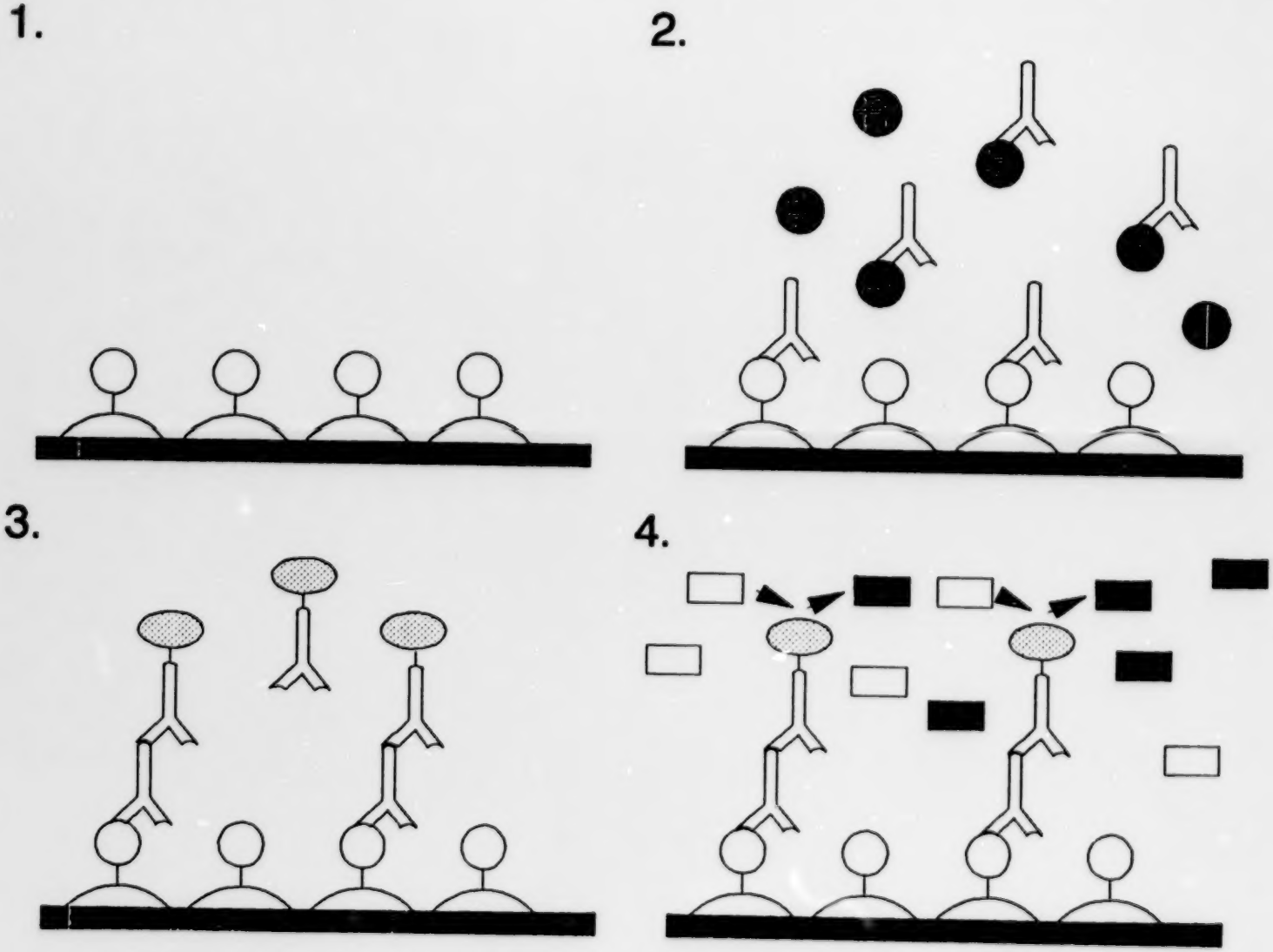


Figure 1. Indirect ELISA. 1. A Polystyrene surface (——) is coated with hapten (♀) linked to carrier protein (◠). 2. Immobilized hapten (♀) and analyte (●) compete for binding to antibody (⅄). 3. A second antibody labelled with an enzyme (⅄) binds to the first antibody. 4. Substrate for the enzyme (□) is added resulting in a colored end product (■) which is measured spectrophotometrically.

known as a goat anti-rabbit antibody. When added to each well of the microtiter plate, the second antibody attaches to the herbicide antibodies. To each goat anti-rabbit antibody an enzyme, such as alkaline phosphatase, has been attached.

After one hour each well is washed three times with a buffer solution to remove any excess goat anti-rabbit antibodies. The wells are then filled with a clear solution containing a chromogenic chemical which turns yellow in the presence of the enzyme. The intensity of the colour in each well can be quantitatively measured using a spectrophotometer.

The presence of free herbicide contained in known standard solutions or in crude well water or plant extracts can be measured using the indirect ELISA procedure. If the herbicide antibody present in the rabbit serum is mixed with a sample containing free herbicide prior to being added to the coated microtiter well, the free herbicide will bind to the specific antibodies in solution and prevent their subsequent binding, by competitive inhibition, to the herbicide/RSA attached to the plate. The result is a decrease in the colour intensity when the chromogenic agent is added to each well since fewer herbicide antibodies will be bound to the herbicide/RSA; subsequently, fewer goat anti-rabbit antibodies to which the chromogenic enzyme is attached will bind to the herbicide antibody. Thus, the intensity of the final colour in each well is inversely proportional the concentration of free herbicide added to the well; that is, the more free herbicide that is added, the less intense is the colour reaction.

Direct ELISA (Fig. 2)

Direct ELISA procedures follow the same general protocol as indirect assays except the procedure is much more simple and rapid. Instead of using a goat anti-rabbit antibody conjugated to an enzyme, the antigen, which is a herbicide in this case, is covalently coupled to the enzyme of choice. The assay is then performed by passively binding the antibody to a microtiter plate after which a solution containing the herbicide/enzyme conjugate and free herbicide are added to each well of the microtiter plate (Fig. 2). After completion of the binding the wells are washed and the substrate added. The intensity of the color reaction is inversely proportional to the concentration of the free herbicide.

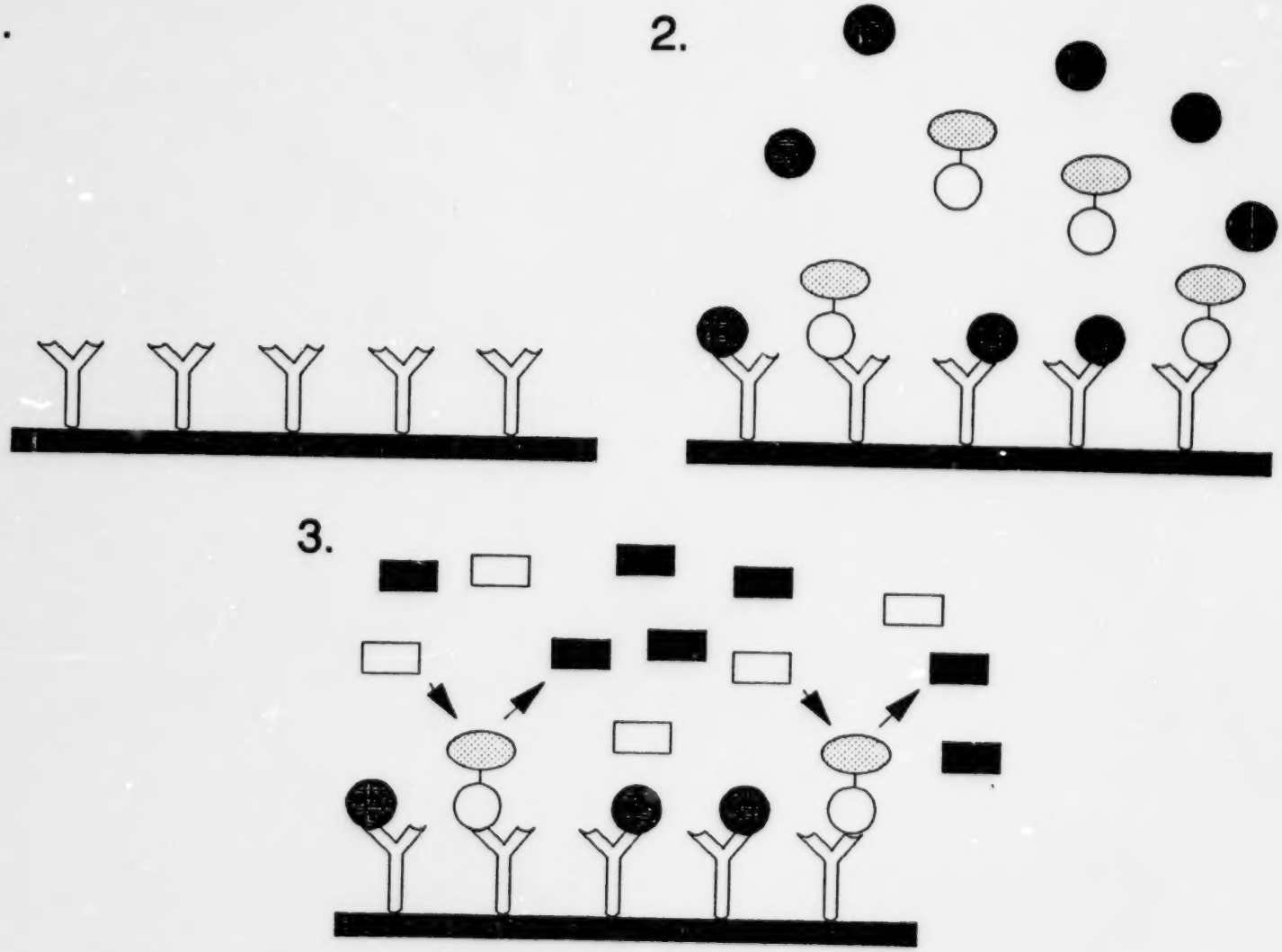


Figure 2. Direct ELISA. 1. A polystyrene surface (——) is coated with antibody (Y). 2. Analyte (●) and enzyme-linked hapten ( ) compete for binding to the antibody. 3. Substrate for the enzyme (□) is added resulting in a colored end product (■) which is measured sprectrophotometrically.

Radioimmunoassay (Fig. 3)

The RIA procedure is much simpler than the ELISA procedure. However, this technique can be used only in laboratories licensed to use radioisotopes. The principles of an RIA are similar to those of the ELISA. A buffer solution containing a known amount of radioactive herbicide is incubated for two hours with the respective sera containing the antibodies to the herbicide (Fig. 3). During this time the radioactive herbicide becomes attached to the herbicide antibody. Ammonium sulfate is then added to precipitate all protein in the solution. The precipitate is athen pelleted by centrifugation. The radioactive herbicide engulfed by the antibodies also will be contained in the pellet. The solution above the pellet containing the unbound radioactive herbicide is removed and discarded. The protein pellet containing the radioactive herbicide is removed from the test tube and the radioactivity quantified. The amount of free herbicide present in water or plant extracts can then be determined by adding the free herbicide in combination with radioactive herbicide and its antibody. As in the ELISA procedure there will be a competition between the free herbicide and the radioactive herbicide for the antibody. As the concentration of free herbicide increases less radioactive herbicide is bound by the antibody and therefore less radioactivity is present in the protein pellet at the bottom of the test tube.

CREATION OF A STANDARD CURVE FOR DETERMINING UNKNOWN QUANTITIES OF A HERBICIDE

The procedures for making standard curves for ELISA and RIA have been described in detail by Weiler et al. (23). A standard curve for the indirect ELISA procedure is made by plotting the relative absorbance (A/A_o) values against the respective log concentrations (ng/ml) of the herbicide; where A_o = the maximum absorbance value in the absence of the herbicide standard and A = the absorbance value in the presence of a known quantity of herbicide (Fig. 4, ELISA). A standard curve for the RIA procedure is made in a similar manner except the term relative binding (B/B_o) is used; where B_o = the maximum binding of the radioactive herbicide to the antibody in the absence of unlabelled herbicide standard and B = the binding of radiolabelled herbicide in the presence of a known quantity of herbicide (Fig. 4, RIA). Thus the amount of herbicide present

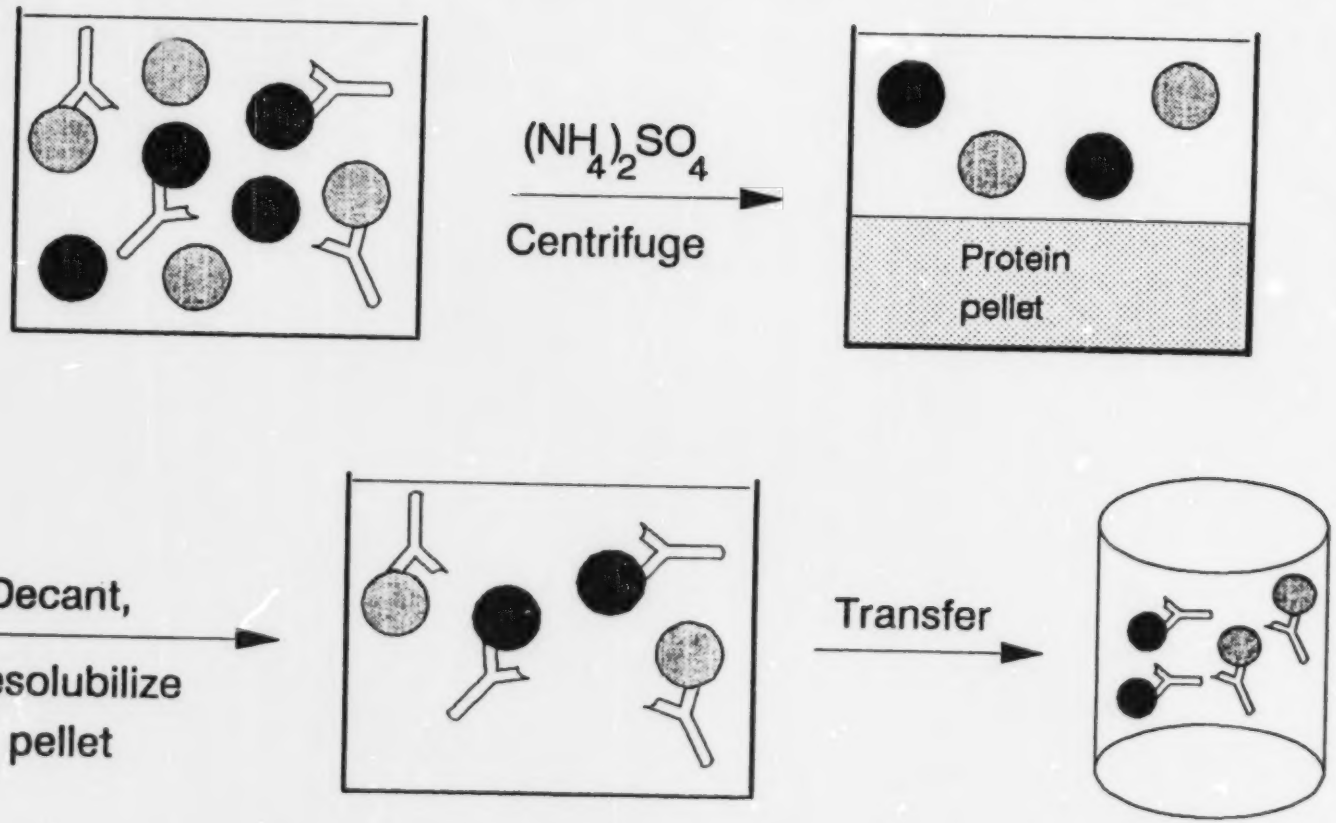


Figure 3. RIA. Analyte (●) and radiolabelled analyte (⊛) compete for binding to the antibody (Y). Ammonium sulfate precipitates the antibody including the bound radiolabelled fraction. The solution is centrifuged to pellet the precipitated antibody. Supernatant, including the non-bound radiolabelled fraction is discarded and the pellet is resolubilized. Radioactivity contained in the resolubilized fraction is determined by liquid scintillation spectroscopy (LSS).

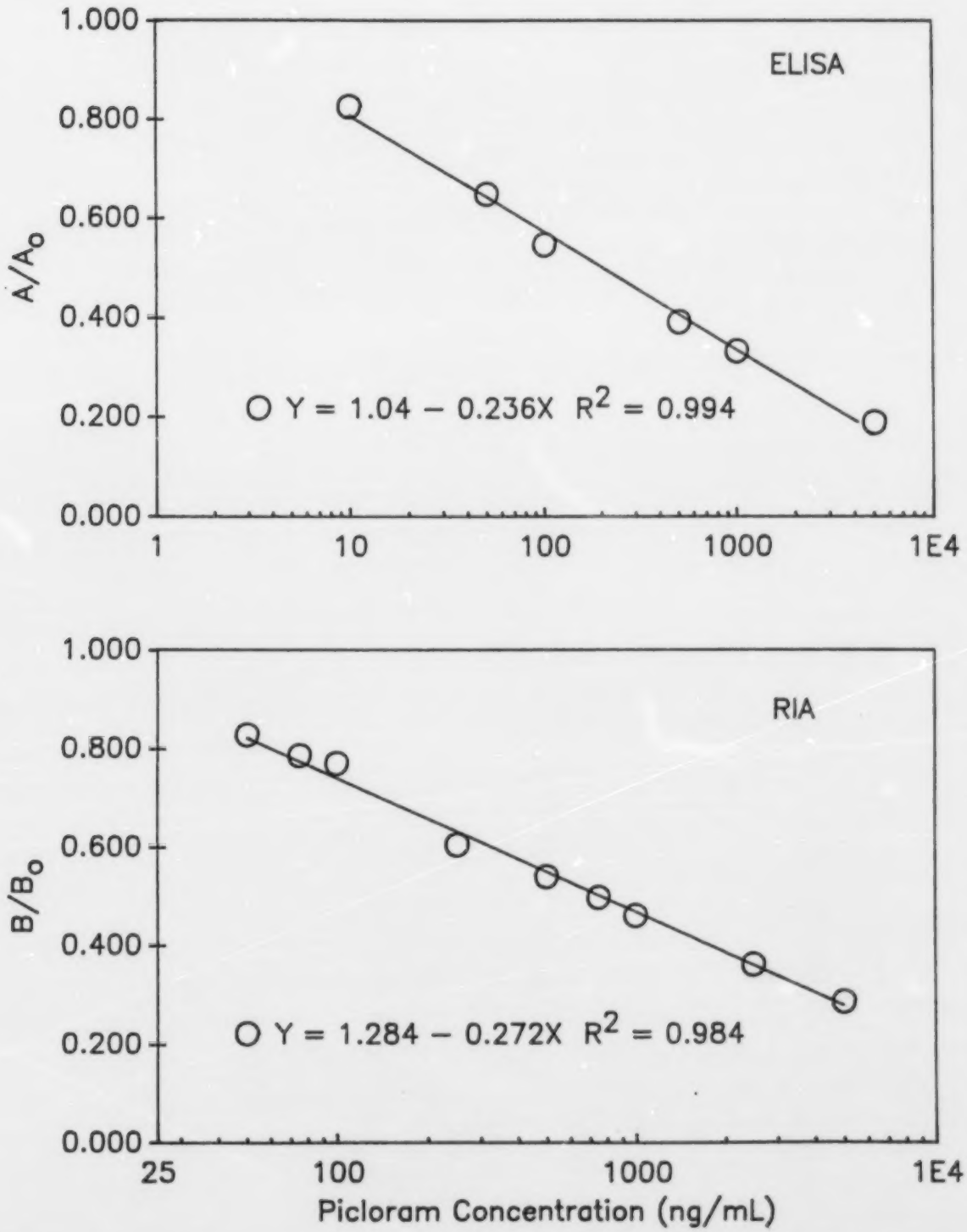


Figure 4. Standard curves for the determination of picloram by ELISA (top) and RIA (bottom). Each point represents the mean of at least four determinations.

in the sample can be determined by interpolation from a standard curve. For example, if we obtained A/A_0 value of 0.6 for an unknown quantity of picloram using the ELISA procedure, this would mean that there was 80 ng/ml of herbicide in the sample (Figure 4, ELISA).

EXAMPLES OF IMMUNOASSAYS DEVELOPED IN OUR LABORATORY

A linear relation between the log of picloram concentration and relative absorbance (A/A_0) was found in the range of 10 to 5,000 ng/mL for the ELISA procedure (Figure 4). A similar relationship was shown between 50 and 5000 ng/mL of picloram (Figure 4) for the RIA procedure. The coefficient of variation within a run was 10% or less for the indirect ELISA method; 3% or less for picloram by the RIA method. Similar standard curves hve been developed for 2,4-D in our laboratory using the indirect ELISA and RIA procedures (6).

Recoveries of 2,4-D and picloram from fortified river water and human urine samples determined by the immunoassays were good with mean overall recoveries varying from 82% to 110% (Table 2). With a concentration step, such as the one described by Fleeker (5) using disposable reverse-phase preparative chromatography columns (octadecylsilane bonded phase packing; C_{18}), the immunoassays also could be applied to studies where a lower limit of detection is required.

To determine the cross reactivity of the 2,4-D antisera with structurally related herbicides, a RIA was conducted whereby binding of radioactivity was inhibited with MCPA, MCPP, 2,4-D, 2,4-DP, 2,4,5-T, and dicamba concentrations of up to 10,000 ng/ml. The cross reactivity of the picloram antisera with 2,4-D and the related pyridine herbicides clopyralid, fluroxypyr, and triclopyr was determined in a similar manner.

MCPA, 2,4,5-T, and 2,4-DP cross-reacted with the 2,4-D antisera to some extent whereas dicamba and MCPP did not (data not shown). The antisera was six times more specific for 2,4-D than for the strongest competitor, MCPA; with 560, 3,600, 5,000, and 10,000 ng/ml of non-labelled 2,4-D, MCPA, 2,4,5-T, and 2,4-DP required for 50% inhibition of binding of radiolabelled 2,4-D, respectively. None of the related pyridine herbicides or 2,4-D inhibited binding of the picloram radiolabel by 50%. The lack of specificity of the picloram antisera

Table 2. Recovery of 2,4-D and picloram from river water and human urine as determined by indirect ELISA or RIA.

Method	Herbicide	Amount of herbicide added (ug/ml)	Recovery[a]	
			River water	Human urine
RIA	2,4-D	0.25	0.21 ± 0.04 (4)	0.25 ± 0.01 (8)
		2.50	2.35 ± 0.04 (4)	2.65 ± 0.15 (7)
ELISA	2,4-D	0.25	0.29 ± 0.06 (2)	0.25 ± 0.03 (9)
		0.75	ND[b]	0.90 ± 0.13 (7)
		2.50	2.44 ± 0.92 (2)	ND
RIA	Picloram	0.25	0.25 ± 0.03 (6)	0.19 ± 0.04 (6)
		2.50	2.60 ± 0.19 (6)	2.22 ± 0.35 (6)

[a] Mean recovery in ug/ml ± standard error (number of determinations)

[b] ND = not determined

for 2,4-D is particularly important since picloram is sold commercially as a mixture with 2,4-D. Therefore, the picloram antisera could be used to quantitate picloram without interference from 2,4-D.

The RIA method was found to be more reliable than the ELISA method. In practice, the RIA was a much simpler procedure requiring fewer steps to complete the assay. The formation of the antibody-antigen complex depends on a combination of weak non-covalent bonds including hydrogen bonds, electrostatic forces, Van der Waals forces, and hydrophobic bonds. Likewise, the passive binding of coating antigen to the microtiter plate well surface depends on these same forces. A successful indirect ELISA requires the formation of these weak bonds at three separate sites; i) between the plate surface and the coating antigen, ii) between the coating antigen and the antibody, and iii) between the antibody and the goat antirabbit-enzyme complex. In comparison, the RIA relies only on the formation of the antibody-antigen complex. It is our opinion that for pesticide determinations, a direct ELISA with monoclonal antibodies specific for the particular herbicide would provide a more simple and reliable assay.

The immunoassays reported here could be incorporated on a routine basis in most laboratories to serve one of two functions. The assays could be used as a rapid, inexpensive method for herbicide quantitation with no sample clean-up. Alternatively, they may be implemented as a preliminary screen to rank samples for follow-up determination by gas chromatography. In either function, the immunoassays represent savings in time, labour, and materials.

Although the ELISA systems described here are workable, there are unavoidable deficiencies in assay performance that are associated with the use of rabbit serum antibodies. These deficencies can be illustrated using 2,4-D as an example. First, upon immunization, the rabbit immune system produces antibodies to three major parts of the 2,4-D/BSA molecule, specifically to: (1) BSA structures, (2) 2,4-D (possibly in one or more spacial configurations) and (3) the combination of 2,4-D and BSA structures. Thus, the amount of specific antibody to 2,4-D that will be ultimately produced by rabbits is limited. Secondly, the binding affinity of the serum antibodies specific for 2,4-D will range from low to high and the serum will display a mean binding affinity. Since

assay sensitivity is directly related to antibody affinity, the sensitivity of an assay utilizing conventional serum antibodies is lower than that which could be achieved if only high affinity antibodies were present. Thirdly, some serum antibodies specific for BSA, 2,4-D/BSA and 2,4-D alone (particularly those with low affinities) can react with closely similar molecules (e.g., other albumins or MCPA which is structurally similar to 2.4-D), thereby reducing the specificity of the assay. Lastly, the quality and quantity of antibodies obtained from rabbit serum vary as the animal ages and is quite variable from rabbit to rabbit. Thus, an ongoing supply of rabbit anti-2,4-D antibodies with consistent high quality performance specifications cannot be achieved. Although some of these deficiencies can be overcome by sophisticated immunochemical techniques, an ELISA or RIA for 2,4-D using rabbit anti-2,4-D serum cannot achieve maximal sensitivity and specificity on an ongoing basis. However, the advent of monoclonal antibodies derived from immortal murine hybridoma cell lines can overcome virtually all of the aforementioned deficiencies and be formatted into a highly specific and sensitive test for 2,4-D.

POLYCLONAL VERSUS MONOCLONAL SYSTEMS

Discussion in this manuscript has centered around the production and use of polyclonal antibody systems. However, it is possible to produce monoclonal antibodies using hybridoma technology. This subject has been thoroughly and concisely reviewed by Russo (19). The advantages and disadvantages of both monoclonal and polyclonal assays as well as the techniques for production of monoclonal antibodies are outlined in Table 3 and Figure 5, respectively.

Russo (19) states that in the production of polyclonal antibodies, the animal will be stimulated to produce many different antibodies to a specific antigen, hence the term polyclonal. This occurs because each plasma cell which is stimulated by the antigen is activated by a different epitopic site on the antigen. In general, the use of serum containing polyclonal antibodies, although easy to produce, has several disadvantages which include: i) variation in serum antibody quantity among animals of the same species, ii) the quantity of specific antibody is minute compared to the other components in the serum, and iii) there is a finite time over which the animal can produce the serum antibodies (i.e.,

Table 3. Comparison of polyclonal and monoclonal antibody assays.

	Polyclonal	Monoclonal
PROJECT COSTS		
Cost of developing reagent	Typically less than $ 1,000	Typically less than $10,000
Time to develop reagent	Minimum 3 months	Minimum 6 months
Expertise required	Injection & bleeding	Injection & bleeding Cell culture skills
Special equipment	None	Cell culture supplies Laminar flow hood CO_2 incubator
REAGENT CHARACTERISTICS		
Chemical structure	Heterogeneous	Homogeneous
Supply	Limited by lifespan of donor animal	Potentially unlimited
Affinity	Heterogeneous population of antibodies that vary in affinity for antigen Antisera has overall average affinity	Can select for clones producing high or low affinity antibody to suit intended use of reagent
Specificity	Usually high, but heterogeneous population may include antibodies that cross react with structurally related antigens	Can select for clones producing high or low affinity to suit intented use of reagent
Sensitivity	Lower detection limit is often in 0.1 to 1 ppb range	Lower detection limit similar to or lower than polyclonal reagent

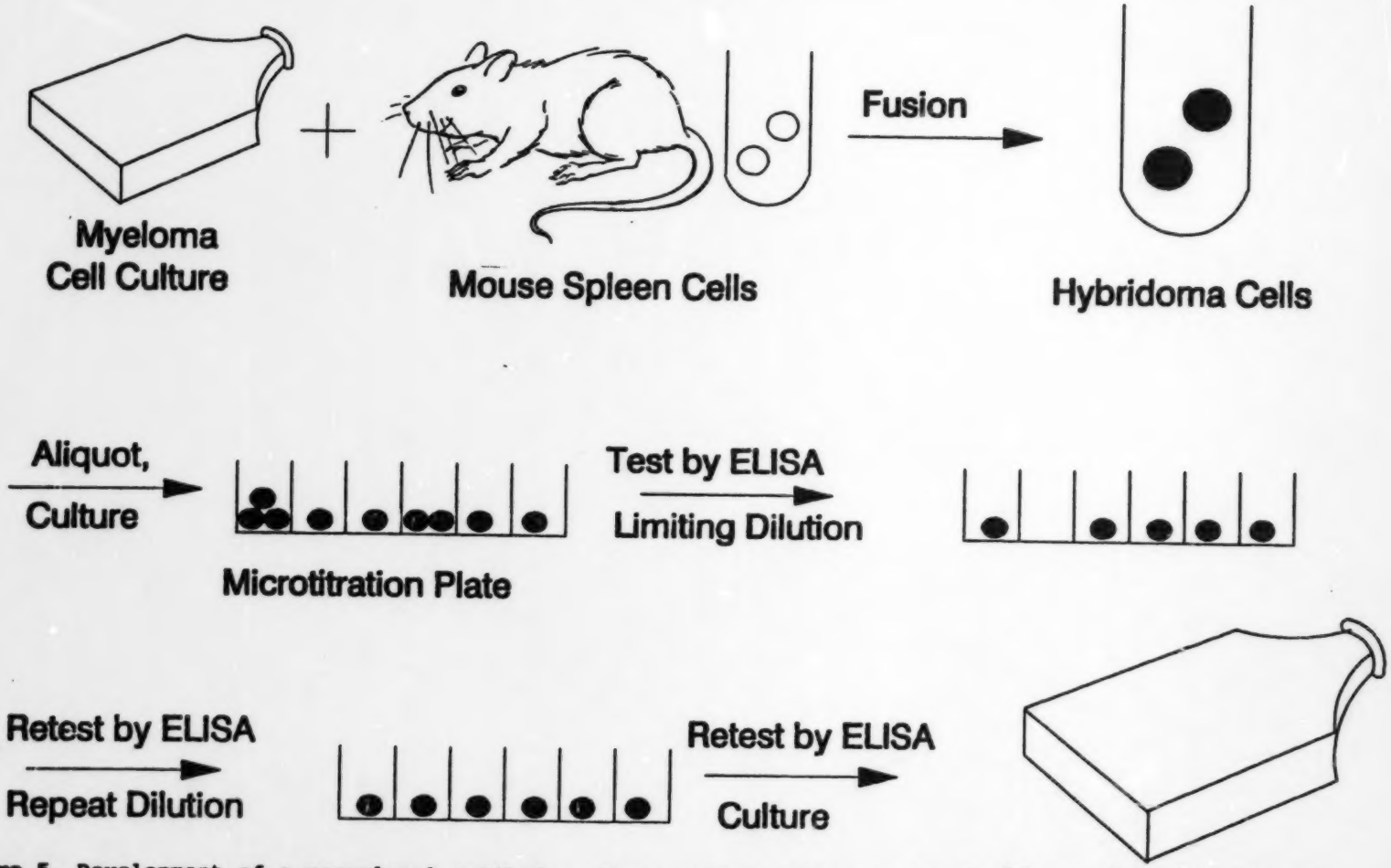


Figure 5. Development of a monoclonal antibody. Mouse spleen cells are fused with mouse myeloma cells to yield immortal hybrid cells which are then cultured. Culture supernatants are tested by ELISA to detect cultures producing the desired antibody. A single cell culture, produced by a limiting dilution process, is cloned and further tested by ELISA to yield an immortal antibody producing cell line.

the animal will eventually die).

A specific monoclonal antibody with specificity for one epitope of an antigen can be produced by fusing spleen cells from an immunized mouse with myeloma cells (tumor cells of B lymphocyte lineage) to produce hybrid cells capable of producing antibodies. These hybrid cells have the genetic immortality of the myeloma cell and can be continually cultured. The hybrid cells, known as hybridomas, still contain a multitude of clones for the antigen and therefore are still polyclonal. However, by performing cloning by limiting dilution a specific clone can be selected. The limiting dilution procedure involves the dilution of a solution of hybridoma cells until only one cell exists per unit of solution. The colony of cells produced by this immortal cell are truly monoclonal. Each monoclonal population is then screened to select the hybridoma population with the desired affinity for a specific epitope on the antigen. This procedure may allow the researcher to select several monoclonal lines that interact with different epitopic sites of the the antigen and/or have different affinities for a common epitopic site of the antigen.

In the fusion process only a small portion of the spleen cells will fuse with myeloma cells. Some cells will not fuse at all and there will be myeloma-myeloma and spleen-spleen cell fusion that also occur. The spleen-spleen cell fusions will die because they lack the capability to continue to grow, however, the myeloma-myeloma fusions will live and grow. Therefore, the myeloma-myeloma cells must be separated from the hybridoma cells. This is accomplished by placing the fused cells in HAT medium which contains aminopterine. The myelomal cells will not grow in the presence of aminopterine because they have a mutation in the gene coding for the enzyme hypoxanthine-guanine-phosphoribosyl-transferase (HGPRT). Therefore, only hybridoma cells which have the normal gene coding for HGPRT enzyme will survive.

The use of monoclonal antibodies will usualy improve the sensitivity and specificity of the direct and indirect ELISA procedures, although the expense and special equipment required for their production may be prohibitive for most weed scientists.

POTENTIAL VALUE TO THE AGRICULTURAL COMMUNITY

Immunoassays offer many advantages over chromatographic procedures currently used to detect and quantitate pesticides. These advantages include speed of processing samples, high specificity for detection of pesticides of interest, reduction in sample preparation and cleanup prior to processing, and dramatic increases in the number of samples that can be processed in a day. Currently, industry, government and universities spend millions of dollars annually in monitoring pesticide residues in potable water, soil, and foodstuffs to ensure these pesticides are used safely and wisely. These agencies are under tremendous time and budget constraints to keep the public informed on the fate of these pesticides. Immunoassay may reduce some of these analysis problems.

Even if immunoassays are not used to accurately quantify levels of pesticides in the environment, they may allow incoming samples to be tested to determine if a pesticide is present in a sample. Since many samples are not contaminated with pesticides, immunoassays can be used to determine which samples are and are not contaminated, thereby allowing all samples testing negative for the presence of pesticide to be eliminated from further testing. All samples testing positive could then be analyzed by chromatographic techniques. This screening could save a great deal of time and money.

In addition, simple diagnostic kits could be made available to farmers and extension representatives for detection of pesticide residues in field situations. For example, a diagnostic kit to determine atrazine levels in soil could be used to determine whether atrazine is present in a field prior to planting a crop that is sensitive to atrazine residues. Water samples from wells and sewers could also be routinely tested on site for pesticides by local authorities. This would save time and reduce costs to farmers and government agencies. Several such diagnostic kits are currently being marketed in the United States for detection of pesticides in water and soil.

LITERATURE CITED

1. Al-Rubae, A.Y. 1978. The enzyme linked immunosorbent assay, a new method for the analysis of pesticide residues. Ph.D. Thesis. The Pennsylvania State University.
2. Cheung, P.K., S.J. Gee, and B.D. Hammock. 1988. Pesticide immunoassay as a biotechnology. In M. Phillips, S.P. Shoemaker, R.D. Middlekauf, and R.M. Ottenbrite, eds. Impact of Chemistry on Biotechnology. American Chemical Society Symposium Series 362; American Chemical Society, Wahington, D.C. pp. 217-229.
3. Ercegovich, C.D., R.P. Vallejo, R.R. Gettig. L. Woods, E.R. Bogus, and R.O. Mumma. 1981. Development of a radioimmunoassay for parathion. J. Agric. Food Chem. 29:559-563.
4. Fatori, D. and W. M. Hunter. 1980. Radioimmunoassay for serum paraquat. Clin. Chim. Acta 100:81.
5. Fleeker, J. 1987. Two enzyme immunoassays to screen for 2,4-dichlorophenoxyacetic acid in water. J. Assoc. Off. Anal. Chem. 70:874-878.
6. Hall, J. C., R. J. A. Deschamps, and K. K. Krieg. 1989. Immunoassays for the detection of 2,4-D and picloram in river water and urine. J. Agric Food Chem. 37:981-984.
7. Hammock, B.D., S.J. Gee, P.K.Y. Cheung, T. Miyamoto, M.H. Goodrow, J. Van Emon, and J.N. Seiber. 1986. Utility of immunoassay in pesticide trace analysis. In R. Greenhalgh and T.R. Roberts, eds. Pesticide Science and Biotechnology. Proceedings ^ International Congress Pesticide Chemistry. Blackwell Scientific Publications. pp. 309-316.
8. Hammock, B.D. and R.O. Mumma. 1980. Potential immunochemical technology for pesticide analysis. In J. Harvey, Jr. and G. Zweig, eds. Recent Advances in Pesticide Analytical Methodology. American Chemical Society Symposium Series 136; American Chemical Society, Washington, D.C. pp. 321-352.

9. Huber, S. J. 1985. Improved solid phase enzyme immunoassay systems in the ppt range for atrazine in fresh water. Chemosphere 14:1795-1803.

10. Huber, S. J. and B. Hock. 1985. A solid-phase enzyme immunoassay for quantitative determination of the herbicide terbutryn. J. Plant Dis. Prot. 92:147-156.

11. Hunter, K.W. and D.E. Lenz. 1982. Detection and quantification of the organophosphate insecticide paraxon by competitive inhibition enzyme immunoassay. Life Sciences 30:355-361.

12. Kelley, M. M., E. W. Zahnow, W. C. Petersen, and S. T. Toy. 1985. Chlorsulfuron determination in soil extracts by enzyme immunoassay. J. Agric. Food Chem. 33:962-965.

13. Knopp, D., P. Nuhn, and H.-J. Dobberkau. 1985. Radioimmunoassay for 2,4-dichlorophenoxyacetic acid. Arch. Toxic. 58:27-32.

14. Levitt, T. 1979. Determinations of paraquat in clinical practice using radioimmunoassay. Proc. Analyt. Div. Chem. Soc. 16:72-76.

15. Mumma, R.O. and J.F. Brady. 1986. Immunological assays for agrochemicals. In R. Greenhalgh, and T.R. Roberts, eds. Pesticide Science and Technology. Proceedings 6 International Congress Pesticide Chemistry. Blackwell Scientific Publications. pp. 341-348.

16. Newsome, W.H. and J.B. Shields. 1981. A radioimmunoassay for benomyl and methyl 2-benzimidazolecarbamate on food crops. J. Agric. Food Chem. 29:220-222.

17. Niewola, Z., C. Hayward, B.A. Symington, and R.T. Robson. 1985. Quantitative estimation of paraquat by enzyme linked immunosorbent assay using a monoclonal antibody. Clin. Chim. Acta 148:149-156.

18. Rinder, D. F. and J. R. Fleeker. 1981. A radioimmunoassay to screen for 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid in surface water. Bull. Environm. Contam. Toxicol. 26:375-380.

19. Russo, A.J. 1988. Immunological assays for the classroom II - hybridoma technology: production of monoclonal antibodies. The American Biology Teacher 50:48-51.

20. Schwalbe, M., E. Dorn, and K. Beyermann. 1984. Enzyme immunoassay and fluoroimmunoassay for the herbicide diclofop-methyl J. Agric. Food Chem. 34:734-741.

21. Van Emon, J., B. D. Hammock, and J. N. Sieber. 1986. Enzyme-linked immunosorbent assay for paraquat and its application to exposure analysis. Anal. Chem. 58:1866-1873.

22. Van Emon, J.M., J.N. Seiber, and B.D. Hammock. 1985. Application of immunoassay to paraquat and other pesticides. In P.A. Hedin, ed. Bioregulators for Pesticide Control. American Chemical Society Symposium Series 276; American Chemical Society, Washington, D.C. pp. 307-316.

23. Weiler, E.W., J. Eberle, R. Mertens, R. Atzorn, M. Feyerbrand, P.S. Jourdan, A. Arnscheidt, and U. Wiecsorek. 1986. Antisera- and monoclonal antibody-based immunoassay of plant hormones. In T.L. Wang, ed. Immunology in Plant Sciences. Society for Experimental Biology Seminar Series 29. Cambridge University Press, Cambridge. pp. 27-58.

Immunoassays for the Detection of 2,4-D and Picloram in River Water and Urine

Abstract. Immunoassays for 2,4-D [(2,4-dichlorophenoxy)acetic acid] and picloram (4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid) detection were developed using polyclonal antibodies raised in New Zealand White rabbits. Concentrations of 2,4-D within the working range of 100 to 10,000 ng/mL and 50 to 10,000 ng/mL could be quantitated with an indirect enzyme-linked immunosorbent assay (ELISA) and a radioimmunoassay (RIA) in river water and urine, respectively. Concentrations of picloram within the working range of 50 to 5000 ng/mL also could be quantitated in river water and urine by RIA. Determinations using the immunoassays required no sample clean-up. Specificities of the antisera for structurally similar herbicides were low compared to 2,4-D or picloram. The RIA methods incorporated a novel radiolabel consisting of [^{3}H]glycine covalently linked to the herbicide molecule. When compared to the ELISA, the RIA was a more simple, efficient and rapid procedure requiring fewer steps to complete the assay. The immunoassays would be suitable for herbicide quantitation in applicator exposure and environmental fate studies.

INTRODUCTION

The potential of immunochemical technology for pesticide analysis has been examined by Hammock and Mumma (1980) and more recently by Van Emon et al. (1985) and by Cheung et al. (1988). Immunoassays are proposed for pesticides that are difficult to analyze by standard techniques. Many pesticides, including 2,4-D and picloram, require an extensive sample preparation including derivatization before they can be analyzed by gas chromatography. As alternative methods, immunoassays can be sensitive, specific, and precise providing for rapid, cost effective analyses.

Current concerns about potential health hazards connected with pesticide use have focused on 2,4-D as a suspected cancer causing agent (Hoar et al., 1986). As a broadleaf weed killer, 2,4-D is used extensively in field crops, on turf, and in non-crop lands. Its widespread use and associated health concerns have made monitoring environmental and biological samples for the

presence of 2,4-D desirable. Among the types of samples monitored are well waters for 2,4-D contamination (Frank et al., 1987) and urine samples for applicator exposure studies (Grover et al., 1986; Libich et al., 1984).

Picloram is used for the control of woody and broadleaf herbaceous plants. It is relatively resistant to breakdown in the environment and has been found to be mobile in the soil (Hamaker et al., 1963). Picloram residues have been found in surface and groundwater samples (Frank et al., 1987; Baur et al., 1972). The mobility in the environment shown by picloram along with the susceptibility of certain crops to extremely small amounts of this compound (Ragab, 1975) make monitoring water for picloram residues necessary.

Radioimmunoassays (RIA) for 2,4-D (Rinder and Fleeker, 1981, Knopp et al., 1985) have been reported. Recently, Fleeker (1987) described two direct enzyme-linked immunosorbent assays (ELISA) developed for the detection of 2,4-D in water. To date, no immunoassays have been reported for picloram. The following report describes the development of an indirect ELISA procedure for 2,4-D and simple RIA procedures for 2,4-D and picloram detection in river water and urine samples without prior clean-up procedures.

MATERIALS AND METHODS

Chemicals and Materials. The analytical standard of picloram and the [2,6-^{14}C]picloram (sp. act. 264 MBq/mmol) were provided by the Dow Chemical Company, Midland, MI. The analytical standard of 2,4-D along with N-hydroxysuccinimide (NHS), N,N'-dicyclohexylcarbodiimide (DCC), 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluenesulfonate (CMC), isobutyl chloroformate, triethylamine, bovine serum albumin (BSA), rabbit serum albumin (RSA), goat antirabbit phosphatase, Sigma 104 phosphatase substrate tablets, Tween 20 (polyoxyethylene sorbitan monolaurate), Freund's complete adjuvant, and Freund's incomplete adjuvant were obtained from Sigma Chemical Company, St. Louis, MO. The ^{14}C-labelled 2,4-D {(2,4-dichlorophenoxy)-[2-^{14}C]acetic acid; sp. act. 11.6 GBq/mmol} was obtained from Amersham/Searle, Don Mills, ON. Aquasol 2 and [2-^{3}H]glycine (sp. act. 1609.5 GBq/mmol) were obtained from New England Nuclear Research Products, Boston, MA. Diethanolamine was obtained from Fisher

Scientific Ltd., Don Mills, ON.

Caution: Precaution is advised when handling the herbicides mentioned in this paper; avoid contact, wear protective clothing, avoid inhalation and work in a fume hood.

Instruments. The optical density of microtiter plate well content was read on a Bio-Rad Model 2550 EIA Reader. Liquid scintillation spectroscopy was performed on a Packard Tri-Carb 460C liquid scintillation system.

Buffers. Phosphate buffered saline (PBS) contained 8.00 g of NaCl, 0.20 g of KH_2PO_4, 2.90 g of Na_3PO_4 $12H_2O$, and 0.50 g of KCl per L of distilled water. The pH was adjusted to 7.4 with 1 M HCl. PBS-Tween washing solution was prepared by adding 0.5 mL of Tween 20 per L of PBS. Diethanolamine buffer contained 100 mL diethanolamine per L of distilled water. The pH was adjusted to 9.8 with 1 M HCl.

Water and Urine Samples. River water was collected from the Speed River, Guelph, Ontario. The water was filtered through a Whatman No. 1 filter paper, and stored at 4°C until time of analysis. Human urine was collected from a male donor over a 24 h period, pooled, and stored at 4°C. Water and urine samples were fortified with an ethanolic solution of analytical standard of 2,4-D or picloram to achieve final sample concentrations within the working range of the immunoassays.

Preparation of Immunogens. Picloram and 2,4-D were conjugated to BSA as described by Fleeker (1987). Equimolar amounts of [^{14}C]picloram (46 mg, 45.5 Bq), NHS (22 mg), and DCC (39 mg) were dissolved in the sequence given in 2.5 mL of dioxane. The solution was allowed to stand at room temperature for approximately 18 h at which time it was filtered to remove the precipitate. The filtrate was evaporated to dryness on a rotary evaporator under vacuum at 35°C. A solution of BSA (500 mg) dissolved in 3 mL of 0.10 M borate buffer (pH 9) was added to the residue and the mixture was agitated gently for 1 h at room temperature. The resulting solution was dialyzed against several changes of deionized water over 36 h at 4°C and lyophilized. The procedure was repeated using [^{14}C]2,4-D (42 mg, 45.5 Bq) in place of picloram. The amount of herbicide bound to BSA was estimated by measuring ^{14}C present in weighed portions of

product dissolved in PBS. Approximately 20 and 15 molecules of picloram and 2,4-D, respectively, were bound per BSA molecule.

Antisera. New Zealand White rabbits were injected subcutaneously with an emulsion consisting of 0.5 to 1.0 mg immunogen dissolved in 0.5 mL of PBS and an equal volume of Freund's complete adjuvant. The injections were repeated 3, 6, and 10 days after the initial injection, substituting Freund's incomplete adjuvant for complete adjuvant. A booster injection was given one month after the initial injection and was repeated at monthly intervals thereafter. The rabbits were bled for antibody titer determinations 10 days after each boost. Antisera for 2,4-D or picloram immunoassay development were prepared from a single bleed in each case.

Preparation of Coating Antigen. To a solution of 50 mg 2,4-D (0.23 mmol) in 2 mL of dioxane was added 50 mg CMC (0.12 mmol). The solution was stirred for 2 h at room temperature. RSA (50 mg) was dissolved in 6 mL of 0.1 M borate buffer (pH 9). The 2,4-D solution was added dropwise to the RSA solution over a period of 15 min. The mixture was stirred for 18 h at 4°C and dialyzed against several changes of deionized water.

Preparation of Radiolabels. The mixed anhydride of 2,4-D was prepared by adding 2,4-D (6 mg), triethylamine (5 uL), and isobutyl chloroformate (5 uL) in the sequence given to 500 uL of dioxane. A portion of the mixed anhydride solution (100 uL) was added to a solution of 100 uL of [^{3}H]glycine (0.1 mCi), 100 uL of dioxane, 100 uL of distilled water, and 2 uL of 2 M NaOH. After 1 h, an additional 2 uL of NaOH was added. The reaction was allowed to proceed for a total of 4 h at room temperature.

The 2,4-D-[^{3}H]glycine conjugate was isolated and purified by TLC. The reaction mixture, [^{3}H]glycine, and 2,4-D were spotted on a silica gel plate (Whatman K5F). The plate was developed in a diethyl ether: petroleum ether: formic acid (70:30:2 v/v/v) solvent system to a 10 cm solvent front. The 2,4-D standard and the unreacted mixed anhydride of 2,4-D were visualized under UV light (R_f 0.82). Fractions of the plate were scraped, eluted with 90% EtOH, and assayed for radioactivity. Three fractions contained appreciable amounts of radioactivity: R_f 0.00 (corresponding to [^{3}H]glycine), R_f 0.47, and R_f 0.76. The

fractions were assayed for binding in an RIA using antisera known to have anti-2,4-D activity. Only the fraction corresponding to R_f 0.47 showed binding. The binding also was shown to be competitively inhibited with free 2,4-D. The fraction chromatographing to R_f 0.47 was therefore assumed to be the 2,4-D-[^{3}H]glycine conjugate.

The mixed anhydride reaction was repeated using picloram in place of 2,4-D. The picloram-[^{3}H]glycine conjugate was isolated and purified by TLC as described above with the exception that a solvent system of 60:40:2 diethyl ether: petroleum ether: formic acid (v/v/v) was used for optimum separation.

ELISA Procedure

1. Microtiter plates were coated by adding 100 uL of coating antigen per well (0.023 mg protein per mL) and incubating for 30 min at room temperature.

2. The plate was emptied and washed once with PBS-Tween (200 uL per well).

3. Unoccupied sites on the polystyrene well surface were blocked by treating with a 5% (w/v) solution of powdered milk in PBS (200 uL per well) for 30 min at room temperature.

4. The plate was emptied and washed two times with PBS-Tween as above.

5. Diluted antiserum (1:1000) was preincubated (15 min) with herbicide standard and sample solutions. Aliquots (100 uL per well) of the preincubated mixture were transfered to the wells of the microtiter plate and incubated for 1 h at room temperature. One column of the plate received no coating and no herbicide in order to determine non-specific binding while another column received diluted antisera only to determine the maximum absorbance reading (B_o).

6. The plate was emptied and washed two times with PBS-Tween.

7. Goat antirabbit phosphatase conjugate diluted in PBS (1:5,000,000) was added (100 uL per well) to the plate. The plate was incubated for 30 min at room temperature.

8. The plate was emptied and washed two times with PBS-Tween.

9. Substrate (1 tablet per 5 mL of diethanolamine buffer) was added to the plate (100 uL per well). Color was allowed to develop for 1 h or until a reading of 0.6 to 0.8 AU was obtained.

10. Absorbance of each well was measured at 405 nm. Absorbance of the standards corrected for non-specific binding was divided by B_o (also corrected for non-specific binding). This value was plotted against the log of herbicide concentration (ng/mL) to construct a standard curve. Concentrations of unknowns were calculated on the basis of the standard curve.

RIA Procedure. The following RIA procedure is a modified version of that described by Weiler et al. (1986).

1. Into 1.5 mL microcentrifuge tubes (Fisher Scientific, Don Mills, ON) was transfered 100 uL of standard or sample. Control tubes received 100 uL of non-fortified sample solution.

2. Incubation mix (300 uL per tube) consisting of one part deionized water, one part inert serum, 12 parts PBS, and sufficient radiolabel to yield 10,000 cpm per assay was added to each tube.

3. Antisera diluted in PBS (1:100 for 2,4-D antisera, 1:600 for picloram antisera) was added to the tubes (100 uL per tube). One set of control tubes did not receive antisera for determination of non-specific binding and a second set of control tubes received antisera only for maximun binding of radiolabel (B_o).

4. The contents of the tubes were mixed thoroughly on a vortex mixer followed by a 2 h incubation at 4°C.

5. The antibody-bound radiolabel fraction was precipitated by adding 0.5 mL of a 90% saturated $(NH_4)_2SO_4$ solution, mixing, and incubating for 1 h at 4°C.

6. The precipitate was centrifuged (12,000 x g) for 5 min and the supernatant was discarded. The pellet was washed once with a 0.5 mL portion of a 50% saturated $(NH_4)_2SO_4$ solution. The tubes were re-centrifuged and the supernatant discarded. The pellet was dissolved in two 300 uL aliquots of deionized water which were transfered to 6 mL scintillation vials. Each vial received 4 mL of scintillation cocktail (Aquasol 2).

7. The scintillation vials were assayed for radioactivity. All results were corrected for non-specific binding. Values for standards were divided by B_o and were plotted against the log of the herbicide concentration (ng/mL). The quantity of the herbicide in the unknown sample was calculated based on the

standard curve.

RESULTS AND DISCUSSION

A linear relation between the log of 2,4-D concentration and relative absorbance (B/B_o) was found in the range of 100 to 10,000 ng/mL for the indirect ELISA procedure (Figure 1A). A similar relationship was shown between 50 and 10,000 ng/mL of 2,4-D (Figure 1B) and between 50 and 5000 ng/mL of picloram (Figure 2) for the RIA procedures. Statistical analyses showed that for each method, the slope of the standard curve remained constant between experimental runs while the elevation of the line was subject to small variations. The coefficient of variation (c.v.) within a run was 7% or less for 2,4-D determined by the indirect ELISA method; 9% or less for 2,4-D by the RIA method; and 3% or less for picloram determined by the RIA method.

Recoveries from fortified river water and human urine samples determined by the immunoassays were good with mean overall recoveries varying from 82% to 110% (Table I). The range of concentrations over which 2,4-D and picloram was accurately quantitated with no sample clean-up correspond with levels found in urine in applicator exposure studies conducted by Libich et al. (1984) as well as with levels reported from environmental fate studies conducted by Thompson et al. (1984) and Hall et al. (1987). With a concentration step, such as the one described by Fleeker (1987) using disposable reverse-phase preperative chromatography columns (octadecylsilane bonded phase packing; C_{18}), the immunoassays also could be applied to well water contamination studies where a lower limit of detection is required (Frank et al., 1987).

To determine the specificity of the antisera for 2,4-D, a RIA was conducted whereby binding of the 2,4-D-[^{3}H]glycine radiolabel was inhibited with structurally similar herbicides at concentrations up to 10,000 ng/mL. The

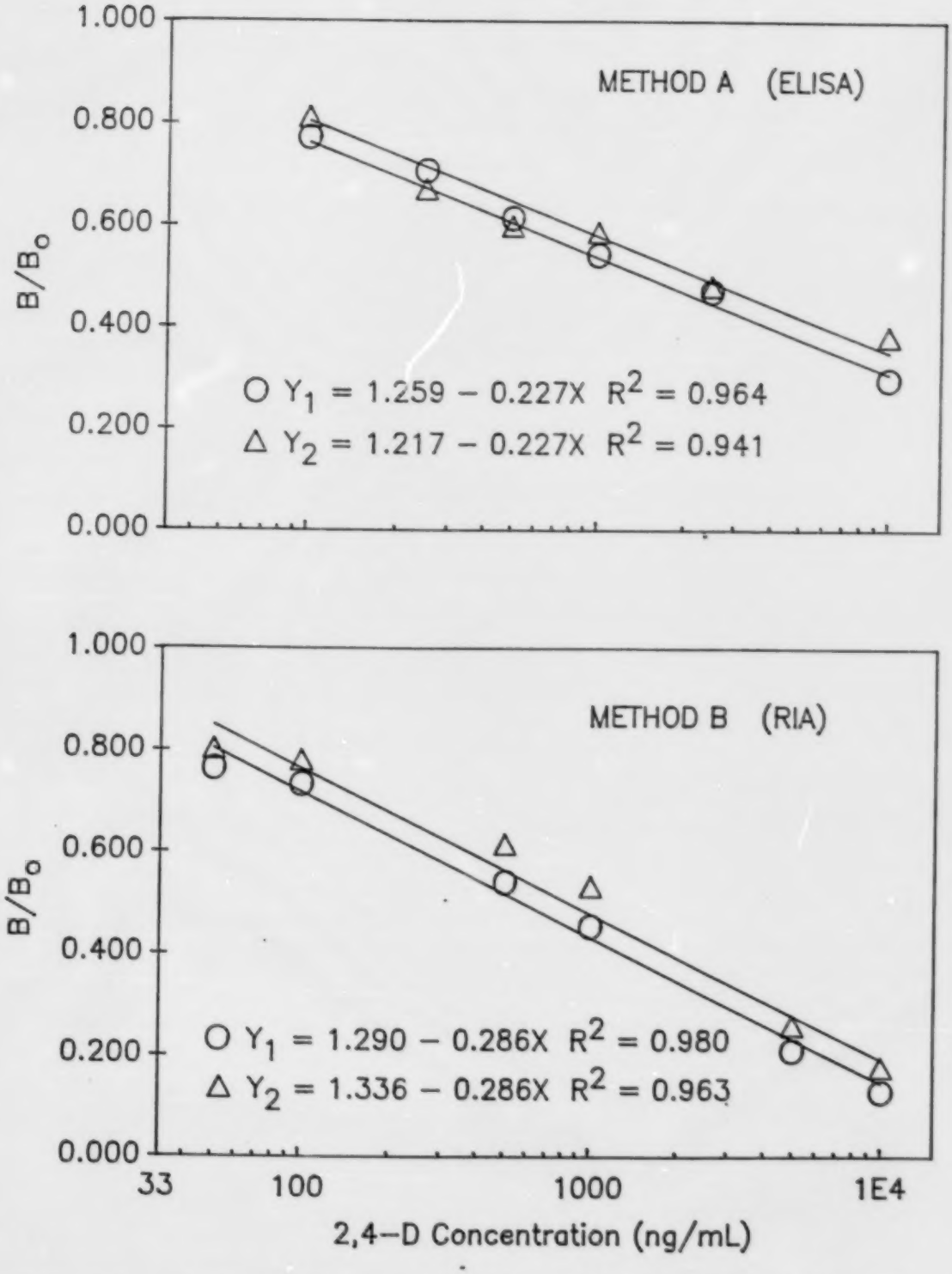


Figure 1. Standard curves for duplicate runs for the determination of 2,4-D by Method A (ELISA) and Method B (RIA). Each point represents the mean of four or five determinations.

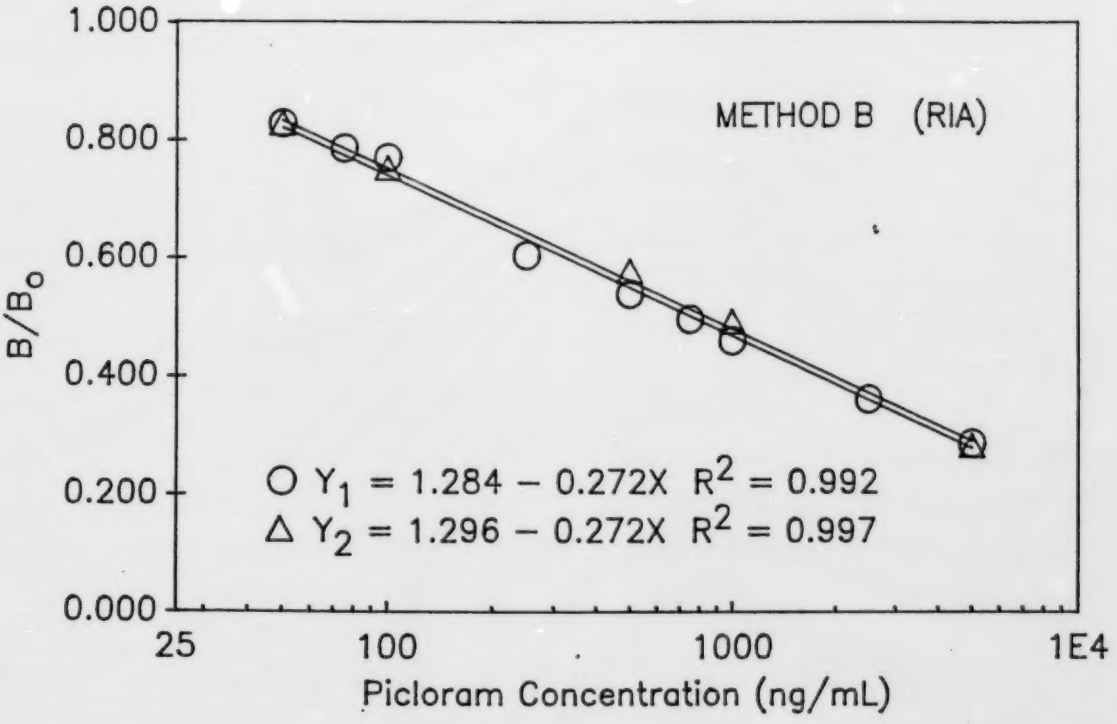


Figure 2. Standard curves for duplicate runs for the determination of picloram by RIA. Each point represents the mean of four or five determinations.

Table I. Recovery of analyte from river water and human urine samples as determined by RIA or indirect ELISA.

Method	Analyte	Amount of analyte added, ug/mL	Recovery[a] River water	Recovery[a] Human urine
RIA	2,4-D	0.25	0.21 ± 0.04 (4)	0.25 ± 0.01 (8)
		2.50	2.35 ± 0.04 (4)	2.65 ± 0.15 (7)
ELISA	2,4-D	0.25	0.29 ± 0.06 (2)	0.25 ± 0.03 (9)
		0.75	ND[b]	0.90 ± 0.13 (7)
		2.50	2.44 ± 0.92 (2)	ND
RIA	picloram	0.25	0.25 ± 0.03 (6)	0.19 ± 0.04 (6)
		2.50	2.60 ± 0.19 (6)	2.22 ± 0.35 (6)

[a]Mean recovery: ug/mL ± SE (number of determinations).

[b]ND = not determined.

specificity of the picloram antisera was determined in a similar manner. The results indicated that 2,4,5-T [(2,4,5-trichlorophenoxy)acetic acid], MCPA [(4-chloro-2-methylphenoxy)acetic acid], and dichlorprop [(±)-2-(2,4-dichlorophenoxy)propanoic acid] cross-reacted with the 2,4-D antisera to some extent (Table II). The antisera was six times more specific for 2,4-D than for the strongest competitor, MCPA. None of the related herbicides tested were able to inhibit binding of the picloram radiolabel by 50%. The lack of specificity of the picloram antisera for 2,4-D is particularly important since picloram is sold commercially as a mixture with 2,4-D.

The RIA methods reported here incorporate a novel radiolabel. Herbicides labelled with ^{14}C are easily obtained but do not lend themselves to sensitive and accurate immunoassay work because of low specific activities (Hammock and Mumma, 1980). Radioimmunoassays utilizing high specific activity radiolabels such as [^{3}H]2,4-D (Knopp et al., 1985) or an [^{125}I]2,4-D derivative (Rinder and Fleeker, 1981) have given good results. Covalently linking the herbicide molecule with [^{3}H]glycine yields a radiolabel with high specific activity without the expense of purchasing a custom synthesized tritiated herbicide or the health hazards connected with iodated radiolabels.

In practice, the RIA was a much simpler procedure requiring fewer steps to complete the assay. The formation of the antibody-antigen complex depends on a combination of weak non-covalent bonds including hydrogen bonds, electrostatic forces, Van der Waals forces, and hydrophobic bonds. Likewise, the passive binding of coating antigen to the microtiter plate well surface depends on these same forces. A successful indirect ELISA requires the formation of these weak bonds at three separate sites: i) between the plate surface and the coating antigen, ii) between the coating antigen and the antibody, and iii) between the antibody and the goat antirabbit-enzyme complex. In comparison, the RIA relies only on the formation of the antibody-antigen complex. Therefore, the RIA was the preferred assay procedure since there is less possibility for the introduction of experimental error. It is our opinion that for pesticide determinations, a direct ELISA with monoclonal antibodies specific for the

Table II. Specificity of antisera for 2,4-D or picloram compared to some structurally similar herbicides determined by the RIA method.

Antisera	Compound[a]	Amount of compound required for 50% inhibition in binding of radiolabel, ng/mL
2,4-D	2,4-D	560
	MCPA	3,600
	2,4,5-T	5,000
	dichlorprop	10,000
	mecoprop	>10,000
	dicamba	>10,000
picloram	picloram	760
	clopyralid	>10,000
	triclopyr	>10,000
	2,4-D	>10,000

[a]2,4-D: (2,4-dichlorophenoxy)acetic acid, MCPA: (4-chloro-2-methylphenoxy)acetic acid, 2,4,5-T: (2,4,5-trichlorophenoxy)acetic acid, dichlorprop: (±)-2-(2,4-dichlorophenoxy)propanoic acid, mecoprop: (±)-2-(4-chlorophenoxy)propanoic acid, dicamba: 3,6-dichloro-2-methoxybenzoic acid, picloram: 4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid, clopyralid: 3,6-dichloro-2-pyridinecarboxylic acid, triclopyr: [(3,5,6-trichloro-2-pyridinyl)oxy]acetic acid.

particular herbicide would provide a more simple and reliable assay compared to the polyclonal indirect ELISA system.

The immunoassays reported here could be incorporated on a routine basis in most laboratories to serve one of two functions. The assays could be used as a rapid, inexpensive method for herbicide quantitation with no sample clean-up. Alternatively, they may be implemented as a preliminary screen to rank samples for follow-up determination by gas chromatography. In either function, the immunoassays represent savings in time, labor, and materials.

LIERATURE CITED

Baur, J. R.; Bovey, R. W.; Merkle, M. G. "Concentration of Picloram in Runoff Water". Weed Sci. 1972, 20, 309-313.

Cheung, P. Y. K.; Gee, S. J.; Hammock, B. D. "Pesticide Immunoassay as a Biotechnology". In Impact of Chemistry on Biotechnology; Phillips, M., Shoemaker, S. P., Middlekauf, R. D., Ottenbrite, R. M., Eds.; ACS Symposium Series 362; American Chemical Society: Washington, DC, 1988; pp 217-229.

Fleeker, J. R. "Two Enzyme Immunoassays to Screen for 2,4-Dichlorophenoxyacetic Acid in Water". J. Assoc. Off. Anal. Chem. 1987, 70, 874-878.

Frank, B.; Clegg, B. S.; Ripley, B. D.; Braun, H. E. "Investigations of Pesticide Contaminations in Rural Wells, 1979-1984, Ontario, Canada". Arch. Environ. Contam. Toxicol. 1987, 16, 9-22.

Grover, R.; Cessna, A. J.; Muir, N. I.; Riedel, D.; Franklin, C. A.; Yoshida, K. "Factors Affecting the Exposure of Ground-Rig Applicators to 2,4-D Dimethylamine Salts". Arch. Environ. Contam. Toxicol. 1986, 15, 677-686.

Hall, J. C.; Bowhey, C. S.; Stephenson, G. R. "Lateral Movement of 2,4-D from Grassy Inclines". Proc. 1987 Brit. Crop Prot. Conf. - Weeds. 1987, 2, 593-599.

Hamaker, J. W.; Johnston, H.; Martin, R. T.; Redemann, C. T. "A Picolinic Acid Derivative: A Plant Growth Regulator". Science 1963, 141, 363.

Hammock, B. D.; Mumma, R. O. "Potential of Immunochemical Technology for Pesticide Analysis". In Recent Advances in Pesticide Analytical Methodology; Harvey, J., Jr., Zweig, G., Eds.; ACS Symposium Series 136; American Chemical Society: Washington, DC, 1980; pp 321-352.

Hoar, S. K.; Blair, A.; Holmes, F. F.; Boysen, C. D.; Robel, R. J.; Hoover, R.; Fraumeni, J. F., Jr. "Agricultural Herbicide Use and Risk of Lymphoma and Soft-Tissue Sarcoma". J. Amer. Med. Assoc. 1986, 256, 1141-1147.

Knopp, D.; Nuhn, P.; Dobberkau, H.-J. "Radioimmunoassay for 2,4-Dichlorophenoxyacetic Acid". Arch. Toxic. 1985, 58, 27-32.

Libich, S.; To, J. C.; Frank, R.; Sirons, G. J. "Occupational Exposure of Herbicide Applicators to Herbicides Used Along Electric Power Transmission Line Right-of-Way". Am. Ind. Hyg. Assoc. J. 1984, 45, 56-62.

Ragab, M. T. H. "Residues of Picloram in Soils and Their Effects on Crops". Can. J. Soil Sci. 1975, 55, 55-59.

Rinder, D. F.; Fleeker, J. R. "A Radioimmunoassay to Screen for 2,4-Dichlorophenoxyacetic Acid and 2,4,5-Trichlorophenoxyacetic Acid in Surface Water". Bull. Environ. Contam. Toxicol. 1981, 26, 375-380.

Thompson, D. G.; Stephenson, G. R.; Sears, M. K. "Persistence, Distribution and Dislodgeable Residues of 2,4-D Following its Application to Turfgrass". Pestic. Sci. 1984, 15, 353-360.

Van Emon, J. M.; Seiber, J. N.; Hammock, B. D. "Application of Immunoassay to Paraquat and Other Pesticides". In Bioregulators for Pest Control; Hedin, P. A., Ed.; ACS Symposium Series 276; American Chemical Society: Washington, DC,

1985; pp 307-316.

Weiler, E. W.; Eberle, J.; Mertens, R.; Atzorn, R.; Feyerbrand, M.; Jourdan, P. S.; Arnscheidt, A.; Wieczorek, U. "Antisera- and Monoclonal Antibody-Based Immunoassay of Plant Hormones". In Immunology in Plant Science; Wang, T. L., Ed.; Cambridge University Press, Cambridge. 1986.

Polyclonal and Monoclonal Enzyme Immunoassays for Picloram Detection in Water, Soil, Plants, and Urine

ABSTRACT Two indirect enzyme immunoassays for picloram (4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid) detection were compared in terms of sensitivity, accuracy, and precision. The assay using a rabbit anti-picloram serum had a linear working range from 5 to 5000 ng/ml with I_{50} ranging from 80 to 200 ng/ml and a lower detection limit of 5 ng/ml. The assay using a monoclonal antibody obtained from a mouse hybridoma cell line yielded a linear working range from 1 to 200 ng/ml with I_{50} values ranging from 8 to 12 ng/ml and a lower detection limit of 1 ng/ml. Neither assay showed appreciable cross-reactivity with the structurally related pyridine herbicides, clopyralid, fluroxypyr, and triclopyr. From the analysis of fortified river water, soil extracts, plant extracts, and urine, the monoclonal antibody-based assay was shown to be more sensitive, more accurate, and more precise than the polyclonal antiserum-based assay.

INTRODUCTION

Immunoassays are considered replacements for, or complements to conventional methods of pesticide residue detection since they can potentially provide quantitative data more quickly and at lower cost than conventional techniques (U. S. Congress, Office of Technology Assessment, 1988). Such immunoassays may be based on polyclonal or monoclonal antibodies. The former is a heterogenous mixture of proteins isolated from serum that represents a variety of antibody molecules of differing specificities and affinities. In contrast, the latter is a homogenous reagent possessing a single antibody specificity and affinity. In a variety of assay systems, either monoclonal or polyclonal antibodies may have certain advantages over the other. For a detailed description and comparison of polyclonal and monoclonal antibodies, the reader is referred to the text by Zola (1987).

The majority of published immunoassay techniques for pesticide detection employ polyclonal antibodies. In a review of immunoassays for agrochemicals, Mumma and Brady (1987) cite 49 assays employing polyclonal antibodies and only

12 employing monoclonal antibodies. The reason for this discrepancy in popularity may be that polyclonal antibody-based assays, at first examination, are easier to develop. Obtaining polyclonal antibodies involves synthesizing an immunogen, immunizing an animal species of choice, and collecting the serum containing the antibodies to be used without further processing or the antibodies may be purified before use. Monoclonal antibodies are obtained by hybridizing the non-immortal antibody producing cells from an immunized mouse or rat with immortal tumor cells, selecting those hybrids producing the antibody of interest, and culturing them for mass production of the antibody.

When used as reagents for the quantitation of pesticide residues, monoclonal antibodies have certain advantages over polyclonal antibodies which include: i) hybrid cells can be cultured indefinitely, either in vivo or in vitro to yield a potentially unlimited supply of homogenous, standardized reagent; ii) during the hybridoma selection process, the investigator can select a hybrid cell producing the desired antibodies in terms of specificity and affinity; iii) the monoclonal antibody will be free of antibodies that are specific for irrelevant antigens which may interfere with the assay's performance; and iv) cross-reacitivity with structurally similar molecules (e.g. other members of a pesticide class) can be selected for or against depending upon whether the investigator desires an assay to detect a single pesticide or a class of pesticides (Vanderlaan et al., 1987; Vinas, 1985). Despite these issues which favor monoclonal antibody-based assays, it is possible to develop excellent immunoassays based on polyclonal antibodies.

Earlier, we reported a radioimmunoassay (RIA) procedure for picloram determinations in water and urine employing polyclonal antibodies produced in rabbits (Hall et al., 1989). In the present study, we describe the development of two indirect enzyme immunoassays (EIA); the first using the polyclonal antisera described previously (Hall et al., 1989), and the second using a monoclonal anti-picloram antibody developed in our laboratories. These assays were compared in terms of the characteristics of the standard curves and performance based on the determination of picloram in fortified water, soil extracts, plant extracts, and human urine samples.

MATERIALS AND METHODS

Materials. Analytical standards of picloram, clopyralid (3,6-dichloro-2-pyridinecarboxylic acid), fluroxypyr ([(4-amino-3,5-dichloro-6-fluoro-2-pyridinyl)oxy]acetic acid), and triclopyr ([(3,5,6-trichloro-2-pyridinyl)oxy]acetic acid) along with radiolabelled picloram ([2,6-^{14}C]picloram, sp. act. 264 MBq/mmol) were provided by the Dow Chemical Company, Midland, MI. Female Balb/cJ mice were obtained from The Jackson Laboratory, Bar Harbor, ME, or from Charles River Inc., Montreal, PQ.

Preparation of Immunogen (Fig. 1). The immunogen used for both polyclonal and monoclonal antibody production was the picloram-bovine serum albumin (BSA) conjugate described previously (Hall et al., 1989).

Preparation of Coating Conjugates (Fig 2 and 3). Picloram was conjugated to rabbit serum albumin (RSA) using two different procedures. One procedure yielded a conjugate with peptide linkages between picloram and the protein (RC4) while the other yielded a conjugate with primarily ester linkages (RC5).

RC4. Following a procedure described by Fleeker (1987), equimolar amounts of picloram (46 mg), *N*-hydroxysuccinimide (NHS, 22 mg), and *N,N'*-dicyclohexylcarbodiimide (DCC, 39 mg) were dissolved in 2.5 mL of dioxane. After incubation at room temperature for 18 h, the solution was filtered to remove any precipitate (Fig. 2). The filtrate was dried at 35 °C under vacuum. The residue was resuspended in 3 mL of 0.10 M borate buffer (pH 9.0) containing 500 mg of RSA and was agitated gently for 1 h. The resulting solution was dialyzed against cold flowing tap water for 24 h and lyophilized.

RC5. Picloram (50 mg) was dissolved in 5 mL thionyl chloride ($SOCl_2$) in a small boiling flask (Fig. 3). The solution was refluxed for 2.5 h at 85 °C to form the acid chloride of picloram. Excess thionyl chloride was removed under vacuum at 60 °C on a rotary evaporator. The residue was dissolved in 2 mL of tetrahydrofuran (THF). The picloram acid chloride solution was added slowly with stirring to 200 mg RSA in 10 mL of 0.02 N NaOH. Before the addition of the acid chloride solution was completed, precipitate formed which did not re-solubilize after stirring for 18 h at room temperature. Dilution of the reaction

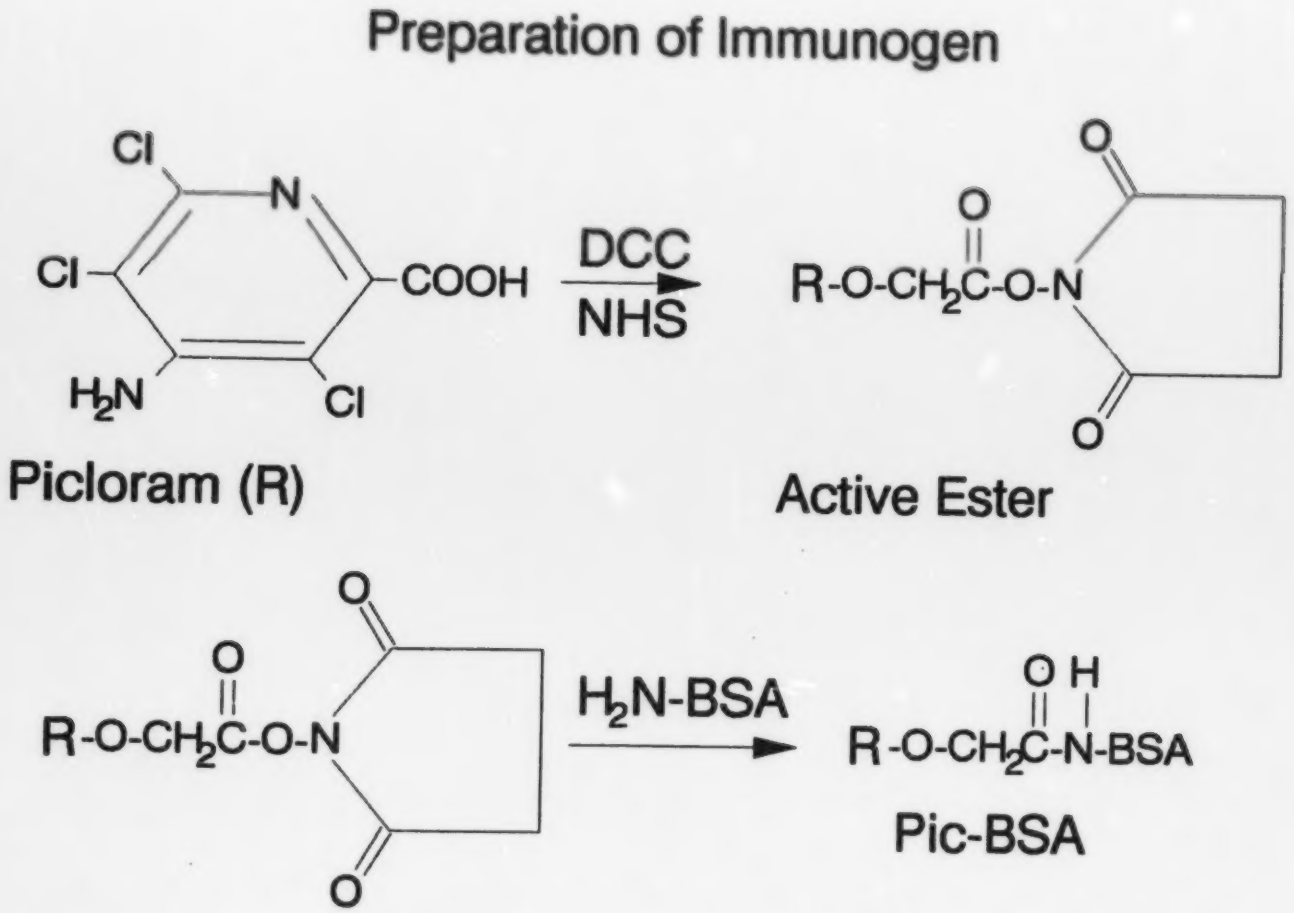

Preparation of Immunogen
Cl
N
Cl
COOH
H_2N
Cl
DCC
NHS
O
O
R-O-CH_2C-O-N
O
Picloram (R)
Active Ester
O
O
R-O-CH_2C-O-N
O
H_2N-BSA
O H
R-O-CH_2C-N-BSA
Pic-BSA

FIGURE 1

Preparation of Coating Conjugate (RC4)

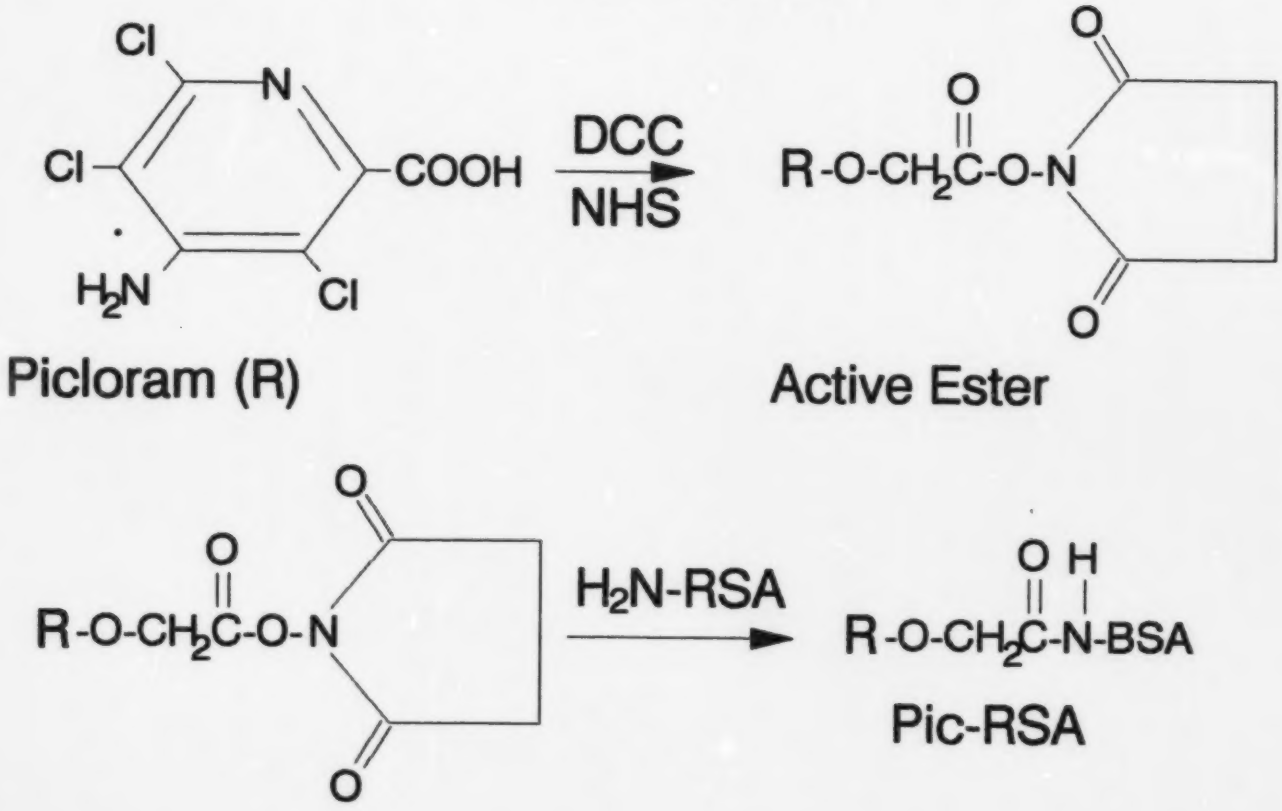
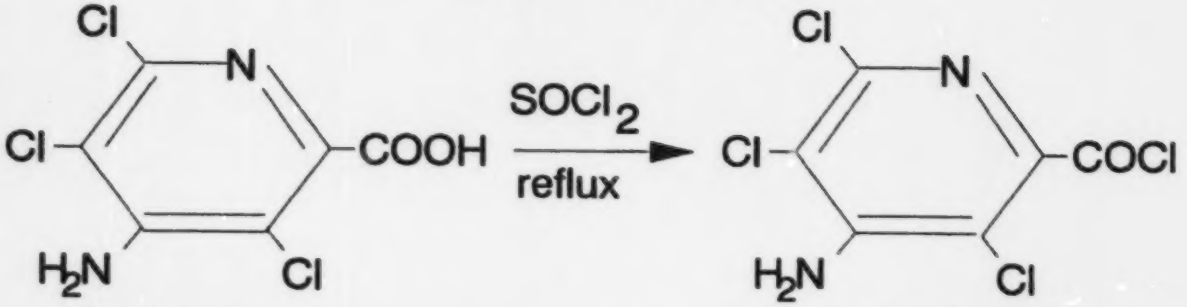


FIGURE 2

Preparation of Coating Conjugate (RC5)



Picloram

Acid Chloride (R-COCl)

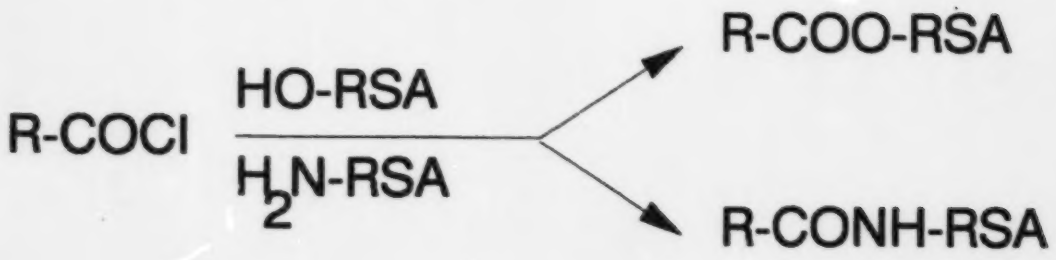


FIGURE 3

mixture to 200 mLs with 0.02 N NaOH succeeded in dissolving most of the precipitate. The resulting suspension was centrifuged to remove any precipitate. The supernatant was dialyzed against cold flowing tap water for 24 h and lyophilized.

Production of Polyclonal Anti-Picloram Antibody. Anti-picloram antisera was obtained from New Zealand White rabbits following the protocol described by Hall et al. (1989).

Production of Monoclonal Anti-Picloram Antibody. Ten 11-week-old mice were injected intraperitoneally with a total volume of 250 uL of a 1:1 (v/v) mixture of 70 ug of immunogen dissolved in phosphate buffered saline (PBS, 0.01 M phosphate, 0.15 M NaCl, pH 7.4) and Freund's complete adjuvant (Difco Laboratories Inc., Detroit, MI). Secondary inoculations were given three and eleven weeks after the initial immunization. One week following each secondary inoculation, the mice were bled from the retro-orbital plexus and the anti-picloram serum antibody titer was determined using the RIA procedure described by Hall et al. (1989). A serum sample was considered positive for anti-picloram antibody activity if binding of the picloram radiolabel was more than twice the level of non-specific binding. After the final secondary inoculation, binding values ranged from 1900 to 4400 fold greater than the level of non-specific binding. Approximately four months after the third injection (six months from the initial immunization), the two mice possessing the highest antisera titers were given a final injection of 200 ug immunogen in 100 uL PBS delivered via a lateral tail vein. Three days later, the mice were sacrificed by cervical dislocation.

The spleen of each mouse was freed of connective tissue and was placed into a Petri dish containing serum-free RPMI medium (Gibco Inc., Burlington, Ontario). The spleen was cut into several small pieces and was gently forced through a 400 mesh stainless steel screen into a second Petri dish which also contained RPMI medium. The cell suspension was transferred to a sterile centrifuge tube and any large tissue aggregates were removed by the sedimentation procedure described by Shortman et al. (1972). The suspension was centrifuged (200 x *g*) for 10 minutes and the cell pellet was resuspended in fresh medium.

Cells in trypan blue viability stain were enumerated microscopically. The spleen cells were mixed with an equal number of SP/2.0 myeloma cells in the semi-log growth phase in RPMI medium. The cell mixture was centrifuged (200 x *g*) for 10 minutes and the cell pellet was suspended in 1 mL of polyethylene glycol (3 000 - 4 000 mol. wt. range) at 37 °C. The suspension was mixed continuously for one minute, followed by the addition of 1 mL of RPMI medium and another one minute of continuous mixing. An additional 9 mL of RPMI medium was added slowly with mixing. The fusion products were centrifuged at 200 x *g* for 10 min, the supernatant discarded, and the cell pellet resuspended in RPMI medium supplemented with 10% fetal bovine serum, 10% NCTC-109 medium and 1% HAT (17). The cell suspension was dispensed (100 uL/well) into six sterile 96 well microtitration plates. The plates were incubated at 37 °C in an atmosphere of 5% CO_2 in air. The fusion procedure was repeated with the second mouse. Four days following the fusion, the cell cultures in the microtitration plates were resupplied with medium by removing 100 uL of supernatant from each well and replacing it with 100 uL of fresh medium. This was repeated daily for three days.

Ten days after the fusion, the cell culture supernatants were screened for the presence of mouse IgG antibodies. Microtitration plates were coated with goat anti-mouse IgG (5 ug/mL, 100 uL/well) diluted in PBS and incubated for 2 h at 37 °C. The plates were washed three times with Tris:Tween (0.02 M tris-HCl, 0.15 M NaCl, 0.05% Tween 20, pH 7.4). The harvested culture supernatant (100 uL) was transferred to the coated plates followed by a further 1 h incubation at 37 °C. After washing the plates with Tris:Tween, goat anti-mouse IgG-alkaline phosphatase conjugate (Zymed Laboratories Inc., South San Fransisco, CA) diluted 1 to 750 in PBS was added and the plates were incubated for 1 h at 37 °C. The plates were washed as before with Tris:Tween and substrate was added (Sigma 104 phosphatase substrate, 1 mg disodium *p*-nitrophenyl phosphate per mL of 1% diethanolamine buffer, pH 9.8). The color reaction was allowed to proceed for 30 min at which time it was stopped with 50 uL/well 2 N NaOH. Absorbance at 405 nm was then determined with a microplate reader. Out of 12 plates, most wells were positive for mouse IgG.

The aforementioned process for detection of IgG in the supernatants was repeated for specific anti-picloram activity by substituting a picloram-RSA coating conjugate (RC4) for goat anti-mouse IgG in the coating step. RC4 had peptide linkages between picloram and the protein molecule. From this assessment, 385 wells from the 12 plates showed a strongly positive anti-picloram activity (Abs_{405} > 1.00). A double screen was conducted on those cultures showing a strongly positive anti-picloram response whereby the culture supernatants were tested for activity against RSA and RC4 coating conjugate in separate sets of plates. Only one culture showed cross-reactivity for RSA and 171 cultures retained a strong anti-RC4 activity. Therefore, of the 171 cultures, 37 were selected to be transferred to 24-well culture plates for further proliferation. The remaining cultures that tested strongly positive against RC4 coating conjugate in both screens (134 cultures) were transferred to sterile 96 well plates to be held in reserve.

After allowing time for the cell cultures in the 24-well culture plates to grow, the EIA assessment was repeated on the culture supernatants. Three cultures were found to be no longer producing antibodies and these were discarded. The remaining cultures were transferred to 25 cm^2 flasks. Subsequent assessment assays in which attempts were made to competitively inhibit binding of the antibodies in the culture supernatant to RC4 coating conjugate with free picloram were unsuccessful. It was postulated that the cultures selected up to this time contained antibodies specific for the peptide link between picloram and RSA in the coating conjugate as no activity was shown against either RSA or picloram alone, although the activity against the coating conjugate was strong. Assessments of the same cultures using a new picloram-RSA coating conjugate (RC5) with primarily ester linkages revealed only one culture with specific anti-picloram activity. Four more cultures containing antibodies specific for picloram were found among the 134 cultures that were held in reserve in 96 well plates. These cultures were screened for cross-reacitivity with three other pyridine herbicides, clopyralid, fluroxypyr, and triclopyr. None of the cultures showed appreciable cross-reactivity with the other pyridine herbicides. Throughout the assessment process, the cultures were gradually depleted of HAT

in the medium by resupplying the cultures with medium containing successively lower amounts of hypoxanthine, aminopterin and thymidine.

The culture showing the best results from the EIA assessment was selected for the limiting dilution procedure to acheive the clonality of the hybridoma cells. The cells were counted in trypan blue viability stain. Based on this figure, dilution factors were calculated to yield ten, five, and one cell per 100 uL of solution. Using a multichannel pipet, 100 uL per well of dilution calculated to yield one cell per well was added to eight columns of a 96 well microtitration plate. Three columns received the dilution calculated to yield five cells per well and the last column was given the dilution calculated to yield ten cells per well. The wells of the plate were checked daily for the presence of a single colony. Once a colony was visible, it was fed with 125 uL of RPMI medium. Supernatant (125 uL) was removed from the well for screening by EIA when the cells of the colony were one-quarter to one-half confluent. Cells from colonies testing positive for anti-picloram antibody activity were transfered to 24 well plates, rescreened by EIA and transfered again into 25 cm^2 flasks if they remained positive for picloram antibodies. The limiting dilution procedure was repeated to ensure monoclonality. After a final assessment by EIA, the cells producing the monoclonal antibodies specific for picloram were collected for the production of ascitic fluid in mice.

Mice were given an injection of 0.5 mL pristane (2,6,10,14-tetramethylpentadecane), a hybridoma growth promoting compound. Seven days later, the mice were injected with 3 x 10^6 hybridoma cells in 200 uL of PBS supplemented with 5% fetal bovine serum. Approximately two weeks following the injection of cells, ascites fluid was withdrawn, centrifuged to remove red blood cells, and frozen at -20 °C until used.

Sample Preparation. Water was collected from the Speed River, Guelph, Ontario and stored at 4 °C. The water was fortified with an acetone solution of picloram. Soil (40 g) was shaken 15 min with 200 mL of a 1:1 methanol/water solution. The mixture was filtered through a glass fibre filter and the methanol was removed under vacuum at 50 °C. The volume of the resulting aqueous solution was returned to 100 mL with Pi buffer (0.1 M phosphate, 1 mM $MgCl_2$, pH 7.5) and

filtered through a 0.45 um nylon filter. The filtered extract solution was fortified with an acetone solution of picloram. Grass clippings (20 g) were homogenized in 100 mL of 0.1 N KOH with 10% KCl. The homogenate was shaken for 30 min and filtered through a glass fibre filter. The filtrate was acidified to pH 2 with 3 N H_2SO_4, refrigerated at 4 °C for 30 min and centrifuged at 3000 x *g* for 10 min. The volume of the supernatant was made up to 100 mL with Pi buffer and aliquots were fortified with an acetone solution of picloram. Prior to analysis, 10.00 mL of the fortified solution was forced through a C_{18} reversed phase liquid chromatography column. The column was washed with 5 mL of water and dried with a gentle stream of forced air for 1 min. The column was eluted with 9 mL of methanol. The eluate was evaporated to dryness and the residue was redissolved in 10.00 mL of Pi buffer. Human urine was fortified with picloram and 10 mL aliquots were acidified to pH 2 with 3 N H_2SO_4. The picloram was extracted three times with 3 mL portions of diethyl ether. The ether fractions were pooled and evaporated to dryness. The residue was redissolved in 10 mL Pi buffer, and cetrifuged at 12 000 x *g* for 10 min.

Recoveries for the extractions described above were determined using [^{14}C]picloram added to soil, grass clippings, and urine, respectively. Recoveries were 95% for the soil extraction, 90% for the plant extraction and 90% for the urine extraction.

Indirect Enzyme Immunoassay. The following procedure is a modified version of that described by Hall et al. (1989).

Microtitration plates were coated by adding to each well 100 uL of RC5 coating conjugate dissolved in Pi buffer (0.1 ug coating antigen per mL). The plates were incubated overnight at 4 °C. The plates were emptied and washed three times with washing solution (Pi buffer supplemented with 0.1% Tween 20). If the plates were not to be used immediately, they were wrapped with plastic and and stored at 4 °C for up to 24 h.

Sites on the polystyrene well surface unoccupied by coating conjugate were blocked by adding 200 uL of 0.1% (w/v) gelatin solution in Pi buffer and incubated for 20 min at 4 °C. The plates were emptied and washed as described above.

Antiserum diluted 1 to 20 000 or ascites fluid diluted 1 to 10 000 in Pi buffer supplemented with 0.05% Tween 20 surfactant (5) were preincubated 1:1 (v/v) with picloram standard or sample solutions. Aliquots of the preincubated mixture were transfered to the wells of the microtitration plate (200 uL per well). The plates were incubated for 1 h at 4 °C.

After washing the plates as before, 200 uL of goat anti-rabbit or goat anti-mouse IgG-horseradish peroxidase conjugate diluted 1 to 5000 in Pi buffer was added to each well and the plates were incubated for 1 h at 4 °C, emptied and washed.

Substrate (1 mg/mL ABTS, 1 mg/mL urea hydrogen peroxide in citrate buffer: 0.024 M citrate, 0.047 M phosphate, pH 5.0) was added and color was allowed to develop for 30 min. The color reaction was stopped by the addition of 100 uL 0.5 M citric acid. Absorbance of each well was measured at 405 nm with a microtitre plate reader. Absorbance values of the standards and the samples (A) were divided by the maximum absorbance value (A_o) representing those wells in which binding of antibody to the coating conjugate was not challenged with free picloram in solution. The A/A_o values for standards were plotted against the log of picloram concentration to construct a standard curve. Concentrations of samples were calculated on the basis of the standard curve.

RESULTS

Enzyme Immunoassay Standard Curves. Picloram standards in Pi buffer were used to generate standard curves for both immunoassays. A linear relation between the log of picloram concentration and relative absorbance (A/A_o) was found in the range 5 to to 5000 ng/mL for the polyclonal assay and 1 to 200 ng/mL for the monoclonal assay (Table I). The monoclonal assay, therefore, had a standard curve with a much steeper slope compared to the polyclonal assay. Typical coefficient of determination values (r^2) were 0.97 for the monoclonal assay and 0.95 for the polyclonal assay.

Assay Sensitivity. The polyclonal assay had an I_{50} value in the range 80 to 200 ng/mL with a lower detection limit of 5 ng/mL (Table I). The monoclonal assay was more sensitive with an I_{50} value ranging between 8 and 12 ng/mL and a

Table I. Characteristics of Indirect Enzyme Immunoassays Using Polyclonal or Monoclonal Anti-Picloram Antibodies.

	PcAb[a]	McAb[b]
I_{50}[c]	80 to 200 ng/mL	8 to 12 ng/mL
Linear range of std curve	5 to 5000 ng/mL	1 to 200 ng/mL
Lower detection limit	5 ng/mL	1 ng/mL

[a]Polyclonal anti-picloram antibody.

[b]Monoclonal anti-picloram antibody.

[c]Concentration of picloram required to inhibit binding of antibody to immobilized coating conjugate by 50%.

lower detection limit of 1 ng/mL. Both assays were more sensitive than the RIA for picloram reported by Hall et al. (2) which had an I_{50} value of 760 ng/mL and a lower detection limit of 50 ng/mL.

Assay Precision. Using the absorbance values of the picloram standards, the interwell variability was determined for the two EIA procedures (Table II). The polyclonal assay showed a mean interwell coefficient of variation (CV) of 6.4% over the standard curve. The mean interwell CV over the standard curve for the monoclonal assay was slightly lower at 5.3%. Interassay CV of the picloram standard A/A_o values determined on four separate runs for the polyclonal assay ranged from 2.1 to 23% with a mean of 12.8% (Table III). For the monoclonal assay, the interassay CV of A/A_o values determined on seven separate occasions ranged from 5.1 to 26% with a mean of 16%. In both cases, CV values increased with an increase in picloram standard concentration due to decreasing A/A_o values. Singh et al. (9) showed similar results for their enzyme immunoassay for the antibiotic sulfamethazine. Intraassay CV values were obtained on picloram determinations in four fortified plant extract samples (Table IV). The polyclonal assay showed a much higher variability with a mean CV value of 80% over the four plant extract samples compared to only 19% for the monoclonal assay over the same samples.

Specificity of the Antibodies. Three structurally related pyridine herbicides, clopyralid, fluroxypyr, and triclopyr were tested for cross-reactivity with the polyclonal and monoclonal anti-picloram antibodies (Table V). Neither antibody cross-reacted appreciably with the other pyridine herbicides as the I_{50} values in all cases were greater than the highest concentration of herbicide tested (50 000 ng/mL for the polyclonal antibody, 10 000 ng/mL for the monoclonal antibody).

Determination of Picloram in Water, Soil Extract, Plant Extract, and Urine Samples. Recovery of picloram from water (Table VI), soil extracts (Table VII), plant extracts (Table VIII), and urine (Table IX) indicated that the monoclonal assay was far superior for quantitative determinations. Overall recoveries for the monoclonal assay were 78, 73, 112, and 167% for water, soil extract, plant extract, and urine, respectively. For the polyclonal assay, overall recoveries

Table II. Interwell Variability of Indirect Enzyme Immunoassay Standard Curve Using Polyclonal or Monoclonal Antibodies.

Antibody	Picloram std, ng/mL	Absorbance ± SE	CV, %
Polyclonal	5	0.715 ± 0.007	3.2
	10	0.676 ± 0.010	4.9
	50	0.606 ± 0.006	3.3
	100	0.580 ± 0.011	6.6
	500	0.505 ± 0.014	9.4
	1000	0.472 ± 0.011	7.8
	5000	0.465 ± 0.013	10
Monoclonal	1	0.797 ± 0.010	4.2
	5	0.637 ± 0.006	3.4
	10	0.484 ± 0.007	5.4
	50	0.261 ± 0.006	7.5
	100	0.185 ± 0.003	5.2
	200	0.154 ± 0.003	5.9

Table III. Interassay Variability of Indirect Enzyme Immunoassay Standard Curve Using Polyclonal or Monoclonal Antibodies.

Antibody	Picloram std, ng/mL	A/A_0[a] Mean	SE	CV, %
Polyclonal	5	0.797	0.010	2.1
	10	0.768	0.024	6.1
	50	0.565	0.029	10
	100	0.482	0.023	9.5
	500	0.342	0.027	16
	1000	0.250	0.029	23
	5000	0.167	0.022	23
Monoclonal	1	0.916	0.018	5.1
	5	0.685	0.016	6.0
	10	0.512	0.016	8.5
	50	0.178	0.012	17
	100	0.095	0.009	23
	200	0.034	0.010	26

[a] A/A_0 = absorbance of standard / maximum absorbance (i.e. conc. picloram=0).

Table IV. Intraassay Variability of Picloram in Four Fortified Plant Extract Samples from Enzyme Immunoassay Standard Curve using Polyclonal or Monoclonal Antibodies.

Picloram added, ng/mL	Picloram recovered			
	PcAb EIA[a]		McAb EIA[b]	
	mean, ng/mL	CV, %	mean, ng/mL	CV, %
4	9.9	87	3.9	29
20	39	89	24	15
40	99	84	52	21
400	780	59	90[c]	10

[a]Polyclonal antibody enzyme immunoassay.

[b]Monoclonal antibody enzyme immunoassay.

[c]1/5 dilution.

Table V. Specificity of Polyclonal and Monoclonal Anti-Picloram Antibodies for Structurally Related Pyridine Herbicides Determined by Indirect Enzyme Immunoassay.

Compound	Chemical name	I_{50}[a], ng/mL	
		PcAb[b]	McAb[c]
clopyralid	3,6-dichloro-2-pyridinecarboxylic acid	>50 000	>10 000
fluroxypyr	4-amino-3,5-dichloro-2-pyridinyloxy-acetic acid	>50 000	>10 000
triclopyr	3,5,6-trichloro-2-pyridinyloxyacetic acid	>50 000	>10 000

[a]Concentration of compound required to inhibit binding of antibody to immobilized coating conjugate by 50%. For the polyclonal antibody, concentrations of herbicides ranging from 5 to 50 000 ng/mL were used while a concentration range from 10 to 10 000 ng/mL was used for the monoclonal antibody.

[b]Polyclonal anti-picloram antibody.

[c]Monoclonal anti-picloram antibody.

Table VI. Recovery of Picloram from Fortified Water Samples Determined by Enzyme Immunoassay using Polyclonal or Monoclonal Antibodies.

Picloram added, ng/mL	Picloram recovered, ng/mL[a]	
	PcAb EIA[b]	McAb EIA[c]
20	27 ± 5.1 (18)	11 ± 0.98 (12)
200	569 ± 79 (18)	165 ± 7.4 (12)
2000	3590 ± 550 (18)	192 ± 8.0 (12)[d]

[a]Mean ± SE (number of determinations).

[b]Polyclonal antibody enzyme immunoassay.

[c]Monoclonal antibody enzyme immunoassay.

[d]1/10 dilution.

Table VII. Recovery of Picloram from Fortified Soil Extract Samples Determined by Enzyme Immunoassay using Polyclonal or Monoclonal Antibodies.

Picloram added, ng/mL	Picloram recovered, ng/mL[a]	
	PcAb EIA[b]	McAb EIA[c]
4	23 ± 4.4 (24)	2.1 ± 0.15 (36)
20	90 ± 23 (24)	13 ± 0.53 (36)
40	110 ± 23 (24)	33 ± 1.1 (36)
400	1010 ± 280 (24)	97 ± 2.5 (12)[d]

[a]Mean ± SE (number of determinations).

[b]Polyclonal antibody enzyme immunoassay.

[c]Monoclonal antibody enzyme immunoassay.

[d]1/5 dilution.

Table VIII. Recovery of Picloram from Fortified Plant Extract Samples Determined by Enzyme Immunoassay using Polyclonal or Monoclonal Antibodies.

Picloram added, ng/mL	Picloram recovered, ng/mL[a]	
	PcAb EIA[b]	McAb EIA[c]
4	9.9 ± 2.5 (12)	3.5 ± 0.23 (24)
20	39 ± 10 (12)	24 ± 1.0 (24)
40	99 ± 24 (12)	51 ± 2.4 (24)
400	780 ± 130 (12)	90 ± 2.6 (24)[d]

[a]Mean ± SE (number of determinations).

[b]Polyclonal antibody enzyme immunoassay.

[c]Monoclonal antibody enzyme immunoassay.

[d]1/5 dilution.

Table IX. Recovery of Picloram from Fortified Human Urine Samples Determined by Enzyme Immunoassay using a Monoclonal Antibody[a].

Picloram added, ng/mL	Picloram recovered, ng/mL[b] McAb EIA[c]
4	11 ± 0.58 (12)
20	30 ± 2.2 (12)
40	50 ± 2.1 (12)
400	90 ± 2.5 (12)[d]

[a]Polyclonal assay was not successful due to unknown contaminant.

[b]Mean ± SE (number of determinations).

[c]Monoclonal antibody enzyme immunoassay.

[d]1/5 dilution.

were 200, 388, and 221% for water, soil extract, and plant extract. The polyclonal assay for determination of picloram in urine was not successful because of unacceptable interference from an unknown contaminant. Picloram concentration estimates were taken from a standard curve made in Pi buffer. Interference from components of the sample matrix likely accounts for much of the error in the concentration estimates. Such interferences from sample components have been reported by Wie and Hammock (1982).

DISCUSSION

For accuracy of quantitation, a standard curve with a steep slope is desirable. The monoclonal assay had a standard curve with a much steeper slope than the polyclonal assay (Table I) and the advantage gained is illustrated by the mean overall recovery of picloram from various fortified samples: 108% for the monoclonal assay compared to 274% for the polyclonal assay (Tables VI, VII, VIII, IX). The high variability of the picloram determinations from the polyclonal assay (Table IV) can also be attributed to the relatively flat standard curve.

A certain amount of the error in the picloram concentration determinations from both assays is due to the fact that the matrix of the fortified samples and the picloram standard matrix (Pi buffer) differed in chemical composition. In preliminary studies, we have found that if a sample has a higher ionic strength than the solution used for the standards, the picloram concentration of the sample will be consistently overestimated. In the reverse situation, concentrations of the sample will be underestimated. We also found that these effects could be minimized if the antibodies were diluted in Pi buffer supplemented with 0.05% (v/v) Tween 20 as described by Hunter and Lenz (1982). Singh et al. (1989) determined sulfamethazine in swine plasma using a standard curve also based on plasma with excellent accuracy. In our previous study (Hall et al., 1989), the RIA procedure for picloram in water and urine using the same polyclonal antisera as in the EIA described here showed a high degree of accuracy (82 to 110% recovery) when the standard curves were constructed using blank water or urine. We chose not to do this for the present study for the following

reason. Soil or water samples from different geographical regions or urine samples from different subjects will vary widely in composition. Selecting one blank water or soil sample to use for the standard curve would not be appropriate and attempts to obtain an "average" water or soil sample would be difficult. It is evident that this choice had more severe consequences with respect to the polyclonal antibody-based assay than with the monoclonal antibody-based assay. The advantage of a standard curve with a steep slope is that small errors in absorbance values will not translate to large errors in concentration estimates.

One disadvantage of a standard curve with a steep slope is the narrow linear working range (Table I). Rather than making several dilutions of a sample in the hope of obtaining one dilution in the proper range, it may be more efficient to conduct a separate assay with a wide working range to rank samples so that appropriate dilutions can be made with certainty for accurate quantitation by a second assay. The polyclonal system described here would be adequate for the role of ranking samples. Alternatively, one could modify the parameters of the monoclonal assay (e.g., increase the antibody concentration) to achieve a standard curve with a flatter slope and a wider working range.

It is commonly stated that for polyclonal antibody production, the design and the preparation of the immunogen are most critical (Hammock et al., 1987; Hammock and Mumma, 1980; Jung et al., 1989). Several studies have illustrated the influence of hapten structure, bridging groups, immunogen structure, and coating conjugate structure on immunoassay performance (Vallejo et al., 1982; Wie and Hammock, 1984; Wie et al., 1982). The goal of immunogen design and preparation is to maximize the quantity of specific antibodies in the antisera having high affinity for the antigen (analyte). In general, one advantage of the monoclonal technique over polyclonal antibody production is that the antigen does not have to be pure for the purposes of immunization as long as during the screening process, high affinity antibodies specific for the antigen of interest can be distinguished from low affinity antibodies and those that are specific for irrelevant antigens. Likewise, the design of the immunogen used to produce monoclonal antibodies may not be as critical as that required for polyclonal antibody production. An effective screening program will enable the investigator

to select and expand the hybridoma cell clone(s) producing the desired antibody even if such clones are rare. In the present study, the same immunogen that yielded a polyclonal antisera with a low average affinity also yielded a monoclonal antibody of high affinity.

LITERATURE CITED

Fleeker, J. Two Enzyme Immunoassays to Screen for 2,4-Dichlorophenoxyacetic Acid in Water. *J. Assoc. Off. Anal. Chem.* 1987, *70*, 874-878.

Hall, J. C.; Deschamps, R. J. A.; Krieg, K. K. Immunoassays for the Detection of 2,4-D and Picloram in River Water and Urine. *J. Agric. Food Chem.* 1989, *37*, 981-984.

Hammock, B. D.; Gee, S. J.; Cheung, P. Y. K.; Miyamato, T.; Goodrow, M. H.; Van Emon, J. Seiber, J. N. Utility of Immunoassay in Pesticide Trace Analaysis. In *Pesticide Science and Biotechnology*; Greenhalgh, R.; Roberts, T. R., Eds.; Blackwell Scientific Publications: Oxford, 1987, pp 309-316.

Hammock, B. D.; Mumma, R. O. Potdential of Immunochemical Technology for Pesticide Analysis. In *Recent Advances in Pesticide Analytical Methodology*; Harvey, J. Jr.; Zweig, G., Eds.; ACS Symposium series 136; American Chemical Society: Washington, DC, 1980; pp 321-352.

Hunter, K. W., Jr.; Lenz, D. E. Detection and Quantification of the Organophosphate Insecticide Paraoxon by Competitive Inhibition Enzyme Immunoassay. *Life Sci.* 1982, *30*, 355-361.

Jung, F.; Gee, S. J.; Harrison, R. O.; Goodrow, M. H.; Karu, A. E.; Braun, A. L.; Li, Q. X.; Hammock, B. D. Use of Immunochemical Techniques for the Analysis of Pesticides. *Pestic. Sci.* 1989, *26*, 303-317.

Mumma, R. O.; Brady, J. F. Immunological Assays for Agrochemicals. In *Pesticide Science and Biotechnology*; Greenhalgh, R.; Roberts, T. R., Eds., Blackwell Scientific Publications: Oxford, 1987; pp 341-348.

Shortman, K. N., Williams, N., Adams, T. The Separation of Different Classes of Cells from Lymphoid Organs. V. Simple Procedure for the Removal of Cell Debris, Damaged Cells and Erythroid Cells from Lymphoid Cell Suspensions. *J. Immunologic Meth.* 1972, *1*, 273-279.

Singh, P.; Ram, B. P.; Sharkov, N. Enzyme Immunoassay for Screening of Sulfamethazine in Swine. *J. Agric. Food Chem.* **1989**, *37*, 109-114.

U. S. Congress, Office of Technology Assessment, *Pesticide Residues in Food: Technologies for Detection*, OTA-F-398 (Washington, DC: U. S. Government Printing Office, October, 1988).

Vallejo, R. P.; Bogus, E. R.; Mumma, R. O. Effects of Hapten Structure and Bridging Groups on Antisera Specificity in Parathion Imunoasay. *J. Agric. Food Chem.* **1982**, *30*, 572-580.

Vanderlaan, M.; Van Emon, J.; Stanker, L. Monoclonal Antibodies for the Detection of Trace Chemicals. In *Pesticide Science and Biotechnology*; Greenhalgh, R.; Rooberts, T. R., Eds.; Blackwell Scientific Publications: Oxford, 1987; pp 597-602.

Vinas, J. Some Advantages and Problems Arising in the Substitution of Traditional Antisera by Monoclonal Antibodies. *Pure Appl. Chem.* **1985**, *57*, 577-582.

Wie, S. I.; Hammock, B. D. Comparison of Coating and Immunizing Antigen Structure on the Sensitivity and Specificity of Immunoassays for Benzoylphenylourea Insecticides. *J. Agric. Food Chem.* **1984**, *32*, 1294-1301.

Wie, S. I.; Hammock, B. D. Development of Enzyme-Linked Immunosorbent Assays for Residue Analysis of Diflubenzuron and BAY SIR 8514. *J. Agric. Food Chem.* **1982**, *30*, 949-957.

Wie, S. I.; Sylwester, A. P.; Wing, K. D.; Hammock, B. D. Synthesis of Haptens and Potential Radioligands and Development of Antibodies to Insect Growth Regulators Diflubenzuron and BAY SIR 8514. *J. Agric. Food Chem.* **1982**, *30*, 943-948.

Zola, H. *Monoclonal Antibodies: A Manual of Techniques*; CRC Press: Boca Raton, 1987; p 214.